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STUDIES ON THE TOXIN PRODUCTION OF THE SHIGA BACILLI

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PLATES 1 AND 2

CORRECTIONS

Vol. 62, No. 5, November 1, 1935

Page 702, at the bottom of the 3rd column of Table II, for 0.36, read 0.26. This change necessitates the following corrections also.

Page 707, 3rd line from the top, for *the entire amount*, read *much*.

Page 715, 9th line from the top, for *actually occurs*, read *occurs to a great extent*. 12th line from the top, for *was 0.36 mg., and precisely this amount was*, read *was 0.36 mg. without deducting about 0.1 mg. for solubility, whereas 0.26 mg. was*.

---

through Berkefeld candles. Others regarded it as an exotoxin, and they obtained it by growing the bacilli in alkaline broth for a period of 2 to 6 weeks, and then filtering. The various workers obtained, however, the same effect on rabbits with the autolyzed bacilli and the filtrates of broth cultures. It was maintained by Klein (9) that it was possible to produce antitoxins against both kinds of toxin.

In 1920, Olitsky and Kligler (10) described methods with which they main-

tained that they were able to separate an exotoxin and an endotoxin from the Shiga bacillus. The exotoxin was derived from 4 to 7 day old broth cultures filtered through Berkefeld candles. It was heat labile, and produced lesions of the central nervous system in rabbits, without at the same time injuring the intestines. The period of incubation of the broth cultures had an important bearing on the nature of the toxic product, as an incubation for 14 days to 3 weeks leads to autolysis of the bacteria and formation of endotoxin. The pure endotoxin exerted a typical action on the intestinal tract, producing edema, hemorrhages, and ulcerations, particularly in the large intestine. It was, however, difficult to prepare a pure endotoxin. When the bacterial growth on agar was washed off in saline solution, and the killed bacterial bodies were autolyzed for 2 days at 37°C., and filtered through Berkefeld candles, usually both toxins were present in the filtrate. The removal of the exotoxin could be carried out by heating the toxic autolysate at 80°C. for 1 hour. The endotoxin was more heat resistant than the exotoxin. Furthermore they found that the two toxins produced different antitoxins. According to the experiments of Olitsky and Kligler, the exotoxin behaved as a pure neurotoxin, and the endotoxin as a pure enterotoxin. Previous workers had not succeeded in demonstrating the distinct biological difference between the two toxins, and Olitsky and Kligler believed this to be due to the methods usually employed in preparing the toxins. This seemed especially likely in the case of the exotoxin, as the previous authors usually had incubated the broth cultures 2 to 3 weeks.

More recent workers, who have studied the poisons of the Shiga bacillus, have paid little attention to the investigations of Olitsky and Kligler. This is particularly the case with those authors who have studied the toxin production of the "S" and "R" forms. Dudgeon and Hope Simpson (11), as also Sudmerson, Rung, and O'Brien (12), found that the S and R variants of the Shiga bacillus were equally toxic. These authors did not, however, prepare endotoxin and exotoxin according to the methods described by Olitsky and Kligler. In the case of other intestinal rods, on the other hand, the conditions seem to be the opposite, as, according to Ibraham and Schütze (13), the S forms usually are more toxic than the R variants.

In a detailed study on the dissociation of the dysentery bacilli, we (14) described several variants. The different variants could be grouped in three serological types and designated S, R, and R_n. The S forms, which were agglutinated in large clumps in homologous immune serum, and the fine agglutinating R forms, were types previously well known from the publications of Arkwright (15) and others. The R_n forms, on the other hand, have not been observed, as yet, by other workers. They lacked the ability to produce antibodies after inoculation into rabbits, and, from this type, we have not succeeded in pre-

paring useful antigens for complement fixation and agglutination tests. The R_n colonies resembled in some respects the G forms of Hadley and his coworkers (16), but they were not, like the G type, filtrable. The S and R forms of the Shiga bacillus, which differed completely from each other, gave no agglutination or complement fixation with the serum of the R_n variant. Hence we have found it interesting to test the toxicity of these serologically different variants. We have tested both the endotoxin and the exotoxin, following the technic of Olitsky and Kligler, as, *a priori*, one might suppose that any possible difference in toxicity then might appear. Especially we were inclined to believe that the endotoxin might depend upon the antigenic behavior of the variants. The results we obtained deviated so far from those of the American authors, that we have found it necessary to undertake detailed anatomical examinations. In the present paper we will deal with these toxin experiments and the consequent histological studies.

Material and Methods

We have used three different strains of the Shiga bacillus. Two of them were isolated in Norway (Strain Røken, 1921 and Strain Aas, 1931), and one strain was obtained from the Lister Institute in 1920. The origin of this strain is unknown. All had the typical cultural characteristics. There was little or no dissimilarity between the toxins of the different strains, and most of the experiments were therefore carried out with the Aas strain. The cultures were of course not those employed by Olitsky and Kligler.

Before preparing the toxins, the S, R, and R_n strains to be tested were seeded on agar plates. Single colonies were fished in four to five consecutive generations, to ascertain that the variants were pure. Furthermore, the morphological, serological, and other properties were tested before preparing the toxins. After this control of the variants, they were subcultivated on agar slants.

Preparation of Endotoxin.—Agar cultures of the variants, controlled as described above, were seeded on agar plates, diameter 9 cm., and cultivated for 24 hours. The growth was washed off with 7 cc. normal saline for each agar plate. The bacterial emulsions in different tests were as nearly as possible made up to the same density. The bacteria were killed by heating in the water bath for ½ hour at 60°C., and the emulsions of killed bacteria were incubated at 37°C. for 2 days, and then filtered through a Berkefeld candle. The clear yellowish filtrate was tested for sterility, and was never kept more than 1 to 2 days before use.

Preparation of Exotoxin.—The variants were cultivated in 50 cc. broth in large bottles, with a diameter of 10 cm. at the bottom. The bottles were kept in the incubator for 3 to 7 days. Good aeration, according to Olitsky and Kligler,

should favor the production of toxin. The cultures were filtered through Berkefeld candles, tested for sterility, and used the following day.

As culture media we have used agar and broth free of sugar, with a reaction set up at pH 7.4 (in the production meat was used (never Liebig extract) and to this 0.2 per cent sodium phosphate and 1 per cent Parke, Davis peptone was added).

The toxins were tested on rabbits and mice. The rabbits were injected intravenously, and the mice intraperitoneally. In all 300 rabbits and about 150 mice were used. The animals were observed several times every day. Autopsy was carried out as soon as possible after death, and specimens from several organs were removed for histological examinations (brain, spinal cord, heart, large and small intestines, liver, spleen, and kidneys).

Dose	S	R	S	R	S	R	R_n	R_n
0.002 cc.					☐	☐	☐	☐
0.004 "			☐	☐	☐	☐	■	■
0.008 "	☐	☐	☐	☐	☐	☐	■	■
0.016 "	■	■	☐	☐	■	☐	■	■
0.032 "	■	⊠	■	⊠	■	⊠		
0.063 "	■	■	⊠	■	■	■	■	■
0.125 "	■	■	■	■	■	■		
0.25 "	■	■	■	■	■	■	■	■
0.5 "	■	■	■	■	■	■		
1 "	■	■	■	■	■	■	■	■

In the charts each column represents one series of animals tested, one animal per dose. The columns which represent parallel tests are separated from the others by thick lines.

■ Dead within 5 days.

⊠ Sick for some days, but recovered.

☐ No symptoms.

CHART 1. Endotoxin experiments in rabbits.

Great care was taken to use animals of the same weight and of the same breed. In comparing the toxicity of the different variants, it was desirable to use numerous series of animals for each toxin. It was, however, impossible to obtain many rabbits of the same weight and of the same breed at the same time. For this reason we could do no more than test two or three series only on the same day (S, R, and R_n, one series for each).

Comparison of the Toxicity of the Different Variants

Endotoxin.—Chart 1 demonstrates different series in parallel of endotoxin experiments. The first two series (S and R) are parallel and comparable because the emulsions used in preparing the toxins had the same density, and the animals were chosen according to the requirements mentioned above. Those tests in

the charts which are parallel, are separated from the others by heavy lines. In the first two series, the minimal lethal dose is 0.016 cc., both in the S and the R toxin. In the next tests, the dose of 0.032 cc. killed the animal in the case of the S toxin, while the corresponding animal in the R toxin experiment was sick for some days. In the third set of two groups, the minimal lethal dose of the S toxin was 0.016 cc. This dose did not affect the rabbit in the case of the R toxin. 0.032 cc. R toxin caused transient symptoms, the minimal lethal dose being 0.063 cc.

The last two series in Chart 1 represent tests carried out with two R_n variants. The minimal lethal dose was in both cases 0.004 cc., and the R_n type might therefore be considered as being more toxic than the others. However, this difference cannot be stressed. The R_n tests are not parallel with the others, as neither the emulsions nor the animals were compared beforehand. In the work of Chart 2, on the other hand, parallel tests with S, R, and R_n endotoxins in mice were carried out. The S toxin seems to be the most toxic, the R_n toxin is slightly less effective,

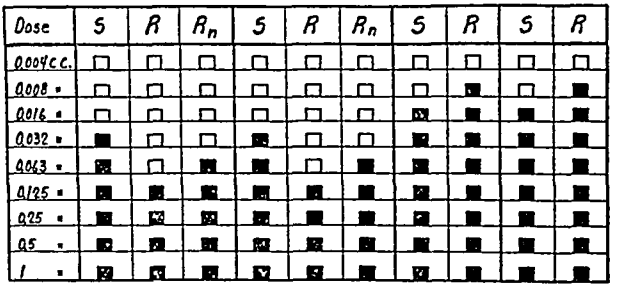

CHART 2. Endotoxin experiments in mice.

while the R is weaker. In the last experiments reported in Chart 2, however, the R toxin proved more efficient than the S. The minimal lethal dose of the R was in both tests 0.008 cc., and in the S 0.016 cc.

There is evidently reason to consider the endotoxins of the three variants S, R, and R_n as about equally toxic. The differences which appeared were small, and due probably to the varying resistance of the animals.

Exotoxin.—Chart 3 gives the results of the exotoxin experiments performed with the S and R variants. In the first two parallel series, the broth cultures had been incubated for 3 days, and the minimal lethal dose of the S toxin was 0.5 cc. The same amount of R toxin caused transient symptoms, while the rabbit receiving 0.25 cc. died. In the remaining parallel tests with cultures 4 to 6 days old, there was no difference in the minimal lethal dose between the S and R exotoxin.

When the experiments in mice are taken into consideration, on the other hand it might be objected that the R exotoxin is more effective than the S. This conclusion is, however, not correct. The mice are extremely variable in their resistance to the toxins of the Shiga bacillus, as Chart 4 shows. In these tests the minimal lethal dose varies from 0.25 cc. to 0.016 cc. with the same toxin.

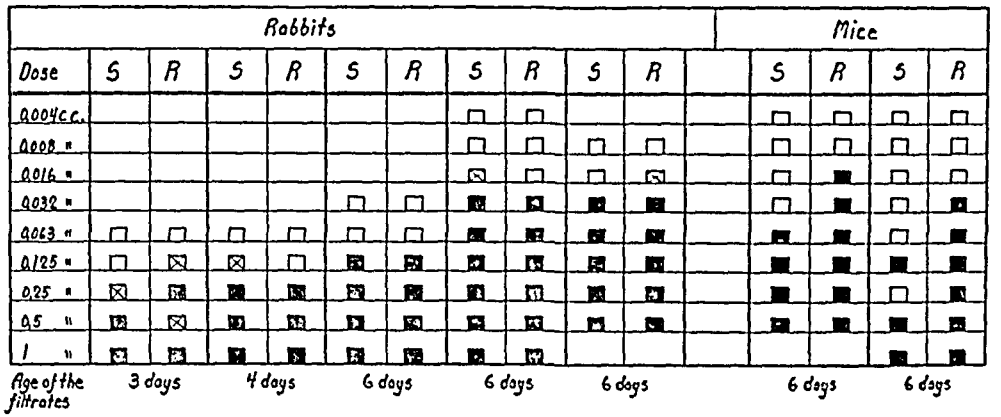

CHART 3. Exotoxin experiments in rabbits and mice.

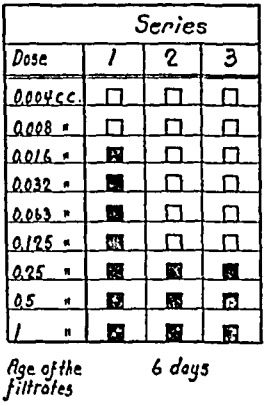

CHART 4. Exotoxin experiments in mice. Three parallel series with one and the same "S" toxin.

These results give ground for the view that the S and the R exotoxins are equally toxic.

We have not found it necessary to carry out serial experiments with R_n exotoxin. A few rabbits only were injected, and the minimal lethal dose seemed to vary from 0.125 cc. to 0.032 cc.

Effect of Endotoxin and Exotoxin upon Rabbits

In testing the toxicity of the different variants we observed symptoms and anatomical changes in the rabbits which were entirely dissimilar to those described by Olitsky and Kligler. For example, we were unable to produce endotoxins which affected the intestinal tract only. The endotoxins which were not heated to 80°C. for 1 hour, injured the intestinal tract and several other organs including the nervous system. Usually the lesions of the nervous system dominated in the clinical picture and postmortem examinations. The unheated endotoxins never failed to affect the spinal cord (Table I).

TABLE I

"S" Endotoxin in Rabbits. Toxin Not Heated

Injected intravenously	Weight	Effect	Histological changes in
cc.	*gm.*		
0.25	1500	Died within 20 hrs.	Intestinal tract, spinal cord, kidneys, and liver
0.125	1725	Died within 30 hrs. Paralysis and diarrhea	Intestinal tract, spinal cord, less in kidneys and liver
0.063	1475	Died within 20 hrs.	" "
0.032	1350	Diarrhea, loss in weight, recovered	
0.016	1400	Died after 48 hrs. Paralysis and diarrhea	Intestinal tract, spinal cord. Kidneys and liver not examined
0.008	1325	" "	" "
0.004	1200	Died within 48 hrs. Paralysis	" "
0.002	1150	Died after 48 hrs. Paralysis	" "

By heating the endotoxins to 80°C. for 1 hour, Olitsky and Kligler succeeded in removing this injurious effect upon the nervous system, though the toxins still were able to affect the intestinal tract. We do not agree with the American authors in this respect. In our hands the heating to 80°C. for 1 hour only attenuated the poisonous effect of the endotoxin (Table II). After heating the endotoxin, the dose of 1 cc. was fatal, while 0.002 cc. caused death before heating. The doses of 0.5, 0.25, and 0.125 cc. caused transient symptoms, while the rest of the rabbits were unaffected. Furthermore as Table II shows, the injurious effect upon both the intestinal tract and the nervous

system is weakened by heating the endotoxin. After injection of 1 cc. of this heated toxin, the rabbit shows both diarrhea and paralysis of the extremities, and at autopsy we succeeded in demonstrating the typical changes in the spinal cord.

In the experiments summarized in Tables I and II, we are dealing with a very powerful endotoxin, as, before heating, the dose of 0.002 cc. was fatal. Usually our endotoxins were not so powerful (Chart 1). When these less poisonous endotoxins were heated to 80°C. for 1 hour, 5 to 10 cc. could be injected intravenously into rabbits, without causing definite symptoms. The rabbits only lost 100 to 150 gm. in

TABLE II

"S" Endotoxin in Rabbits. The Same Toxin as in Table I, Heated at 80°C. for 1 Hour

Injected intravenously	Weight	Effect	Histological changes in
cc.	*gm.*		
1	1700	Died after 24 hrs. Diarrhea and paralysis	Intestinal tract, spinal cord, kidneys, and liver
0.5	1600	Loss of 300 gm. in weight. No distinct symptoms	
0.25	1700	Loss of 300 gm. in weight. Diarrhea, recovered	
0.125	1675	Loss of 100 gm. in weight. No distinct symptoms	
0.063	1375	No symptoms	
0.032	1300	" "	
0.016	1300	" "	

weight. It follows that the rabbits reacted to this inoculation of the heated toxins very well.

Our results in exotoxin experiments do not agree either with those of Olitsky and Kligler. Thus the filtrates of 3, 4, and 6 day old broth cultures affected the central nervous system as well as the intestinal tract (Table III). When the animals survived more than 20 hours, both diarrhea and paralysis of the extremities were observed. The changes in the intestinal tract were as important in the case of the exotoxin as in the endotoxin experiments. On the whole there was no difference between the two toxins. The exotoxin especially always

showed some effect upon the intestinal tract. This was also the case with the filtrates of the young cultures (3 to 4 days).

Turning to the neutralization experiments, the antitoxins produced by inoculating heated endotoxin into rabbits, protected rabbits fairly well against the exotoxin. Table IV demonstrates that S and R anti-endotoxin gave the same protection against S exotoxin. Conversely, the effect of the R exotoxin was distinctly reduced both after neutralization with S and with R anti-endotoxin.

These antitoxins, produced against the heated toxins, were, however,

TABLE III

"S" Exotoxin in Rabbits

Injected intravenously	Weight	Effect	Histological changes in
cc.	*gm.*		
4	1800	Died within 20 hrs.	Intestinal tract, spinal cord, kidneys, less in the liver
1	1625	Died after 48 hrs. Diarrhea and paralysis	Intestinal tract, kidneys, liver. Spinal cord not examined
0.125	1650	Died after 60 hrs. Diarrhea and paralysis	Intestinal tract, spinal cord, kidneys, less in the liver
0.063	1600	Died after 72 hrs. Paralysis	" "
0.032	1400	Loss of 200 gm. in weight. Paralysis, recovered	
0.016	1500	Loss of 100 gm. in weight. Paralysis, recovered	
0.008	1250	No symptoms	

The broth culture was incubated 6 days and then filtered through Berkefeld candle.

not always very powerful, but their efficiency was increased when the inoculation of heated toxins was followed by injections of unheated toxins. As previously mentioned, the animals reacted to the immunization with the heated toxins much better than with the unheated ones. Immunization with unheated toxins or vaccines of the Shiga bacillus will often cause considerable loss in weight or be fatal. When the animals are treated with heated toxins beforehand, they are resistant to prolonged inoculation with toxins which are not detoxicated. The heating of the toxins of the Shiga bacilli has therefore proved to be useful in the production of antibacterial and antitoxic sera.

Anatomical Changes Caused by the Toxins

Organs were removed for histological examination, in order to study the microscopical lesions in the rabbits. The most important injuries were found in the spinal cord and intestinal tract, a fact in correspondence with the clinical picture. We were able, however, to demonstrate lesions in other organs, notably the heart, liver, and kidneys. The spleen and suprarenal glands, on the other hand, were histologically normal.

TABLE IV

Neutralization Experiments with Exotoxin and Anti-Endotoxin

Exotoxin	Anti-endotoxin	Effect
cc.	*cc.*	
0.2 S	0	Died after 24 hrs.
0.2 S	0.1 S	No symptoms
0.2 S	0.01 S	Transient paresis
0.2 S	0.1 R	No symptoms
0.2 S	0.01 R	Transient paresis
0.2 R	0	Died within 24 hrs.
0.2 R	0.1 S	No symptoms
0.2 R	0.01 S	Died within 24 hrs.
0.2 R	0.1 R	No symptoms
0.2 R	0.01 R	No symptoms

The antitoxin was serum derived from a rabbit injected intravenously with endotoxin heated to 80°C. for 1 hour.

The antitoxins were permitted to act upon the toxins for ¾ hour before injecting the rabbits.

0.2 cc. S and R toxin represented 4 to 5 minimal lethal doses.

Nervous System.—Sections from different parts of the brain showed a slight hyperemia, but never hemorrhages. For the rest, the white and gray matter of the brain was intact.

In the spinal cord, including the medulla oblongata, we observed grave lesions. The gray matter, especially in the anterior horns and the motor nuclei of medulla oblongata, was affected to a varying degree. The neurons were partly swollen, with large Nissl bodies (Fig. 1). Sometimes the cells were small, sclerotic, and homogeneously stained (Fig. 2). In the more severe cases, degeneration and necrosis appeared, with result that a few degenerated cells only were visible (Fig. 3). Hyperemia was not usually observed, and hemorrhages only once.

These lesions could be demonstrated in all parts of the spinal cord, but to a varying degree in the different regions.

Olitsky and Kligler have pointed out, however, that hemorrhages in the nervous tissue are regularly present after injection of exotoxin.

Intestinal Tract.—In the small intestines necrosis of the superficial cells was noted. A varying number of leucocytes invaded the mucosa, and the glandular elements were destroyed. Usually the walls of the gut were somewhat congested (Fig. 4).

The large intestines, particularly the cecum and appendix, were affected to a varying degree. The walls of appendix and cecum were thickened, edematous, and greatly congested. Very often more or less extended hemorrhages were associated with the edema. In the more severe cases superficial necrosis was observed (Fig. 5).

Heart.—The heart showed more or less hyperemia in the muscular wall. In some areas red blood corpuscles were found outside the capillaries (Fig. 6).

Liver.—In some cases we found distinct parenchymatous degeneration of the liver cells. In other cases this degeneration was slight, the peripheral cells of the lobules having a lighter color than the central ones. In a few cases no degeneration at all was noted. Hyperemia was always present, however (Fig. 7).

Kidneys.—The cells in the tubuli contorti were always degenerated. In a few cases we found regular parenchymatous degeneration, the nuclei being indistinctly stained. Usually the protoplasm of these cells appeared unstained, so that they acquired an appearance somewhat similar to fatty degeneration. Hyperemia was observed in the kidneys (Fig. 8).

The anatomical lesions described above were found in rabbits injected with exotoxin and endotoxin as well. Furthermore, it made no difference whether the toxins were derived from S, R, or R_n strains. It was noteworthy that the endotoxin, heated to 80°C. for 1 hour, caused the same anatomical changes in the spinal cord as the exotoxin.

DISCUSSION

We have demonstrated that the three serologically different variants of the Shiga bacillus, S, R, and R_n, are equally toxic and are able to cause the same lesions in rabbits. We must therefore conclude that the poisonous product does not depend upon the antigenic behavior of the types. Hence the toxins may be exotoxic in nature, produced by the living bacterial cells, or they may be endotoxins, derived from a part of the bacterial bodies, being similar in the three variants. The last supposition seems to be the most reasonable, as the toxin can be obtained by autolysis of the bacteria. It may, on the other hand, be

presumed that the Shiga bacilli, during their growth on agar surface, are able to secrete the toxin, which will go into solution, when the bacteria are emulsified in saline. Selter (8) has shown, however, that the dead bacterial bodies, which had been used in producing endotoxin, were still toxic after autolysis. Furthermore he succeeded in extracting the same toxin several times from the dead bacilli. There is therefore reason to believe that the toxins of the Shiga bacillus are derived from the bacterial bodies themselves, being thus endotoxic in nature.

Finally the question arises of the explanation for the great disagreement between our results and those of Olitsky and Kligler. Table III shows that the two rabbits injected with 0.032 and 0.016 cc. of the exotoxin exhibited symptoms referable to the nervous system only. It might be concluded that we are here dealing with the pure neurotoxin of Olitsky and Kligler. However, the second and third rabbit of Table III, injected with 1 cc. and 0.125 cc. respectively, showed, on the other hand, diarrhea and paralysis, and the anatomical changes in the intestines were important. The fourth rabbit, receiving 0.063 cc., had paralysis but no diarrhea. At autopsy the lesions in the intestines were distinct but not important. The small doses thus seem to injure the intestinal tract to a slight degree, while the large doses cause more severe changes there. The same conditions appear in Table I also, and in all our experiments the small amounts of toxin seem to have a slight effect only upon the intestines. We might therefore assume that our small doses correspond to the exotoxin (neurotoxin) of Olitsky and Kligler, and our large doses to the endotoxin (enterotoxin) of the same authors. This explanation cannot be said to give entire satisfaction, especially when we keep in mind the results of our anatomical studies. We are in complete disagreement with the authors mentioned as regards the outcome of neutralization tests.

An important change takes place in the toxin on heating to 80°C. for 1 hour. Its poisonous effect is diminished to a large degree, whereas its immunizing ability is unaltered. This temperature effect may possibly be of practical use.

As regards the anatomical lesions in the spinal cord and large intestine, we are in full agreement with the statements of Vaillard and Dopter. The important changes in the small intestines which we have demonstrated, have previously had but little attention. The degen-

erative processes in the parenchymatous organs are worthy of remark. The liver lesions were rather small and inconstant but in the kidneys, on the other hand, degeneration was a constant phenomenon. There is reason to believe that the marasmus and prostration which the rabbits show after the injection of Shiga toxin are partly due to these changes in the kidneys and the liver. On the whole, the rôle of the kidneys in the course of dysentery should have greater attention. In 1923, Hanssen (17) demonstrated that there was a distinct rise in the total amount of urea in the blood, following regular and uncomplicated cases of dysentery caused by Type III (the Sonne bacillus). A chemical control of this injuring effect upon the kidneys in the toxin experiments, should prove of special interest. Furthermore one should test the effect of heat (80°C. for 1 hour) upon this injuring ability of the toxin.

The toxin of the Shiga dysentery bacillus evidently causes degenerative changes in several organs of rabbits. The lesions are accompanied by hyperemia and sometimes by hemorrhages. In the large intestine the hemorrhages often play an important rôle and dominate the anatomical picture. In other organs the hemorrhages are inconstant and less extensive, while hyperemia is observed often.

CONCLUSION

1. The S, R, and R_n variants of the Shiga bacillus are equally toxic.
2. The effect of the toxin upon rabbits is the same, whether it is derived from filtrates of broth cultures (3 to 6 days old), or is obtained by autolysis of the killed bacteria, grown on agar surface. Rabbits show in both cases prostration, loss in weight, paralysis, and diarrhea.
3. When the toxin is heated to 80°C. for 1 hour, its poisonous effect nearly disappears, but its immunizing ability is unaltered. This heated toxin induces a formation of antitoxin, which can protect against the unheated toxins.
4. The anatomical changes observed in the spinal cord (degeneration of the motor neurons) and in the cecum (hyperemia and hemorrhages) are in agreement with the statements of previous authors. Furthermore, the toxin causes hyperemia and hemorrhages in the heart, hyperemia and degeneration in the kidneys and the liver.

The author is indebted to Dr. Jan Jansen, the Anatomical Institute of the University of Oslo, for valuable assistance in preparing the anatomical sections, and for advice in the study of these sections.

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Rabbit 1-41 injected intravenously with 2 cc. S exotoxin. Died after 6 hours. The section from upper thoracic region of the spinal cord shows the swollen neurons with the large Nissl bodies.

FIG. 2. Rabbit 1-08 injected intravenously with 0.125 cc. S endotoxin (not heated). Died after 30 hours, symptoms: diarrhea and paralysis. The section is from the lower thoracic region of the medulla. Small, sclerotic, and homogenously colored cells are recognizable.

FIG. 3. Rabbit 1-11 injected intravenously with 0.016 cc. S endotoxin (not heated). Died after 60 hours, symptoms: diarrhea and paralysis. The section from the upper lumbar region of the medulla shows the degenerated cells and necrotic areas.

FIG. 4. Rabbit 1-13 injected intravenously with 0.25 cc. R endotoxin (not heated). Died after 20 hours. Section from small intestine. The necrosis of the superficial cells is evident. Several leucocytes have invaded the mucous membrane, and the glandular elements are destroyed.

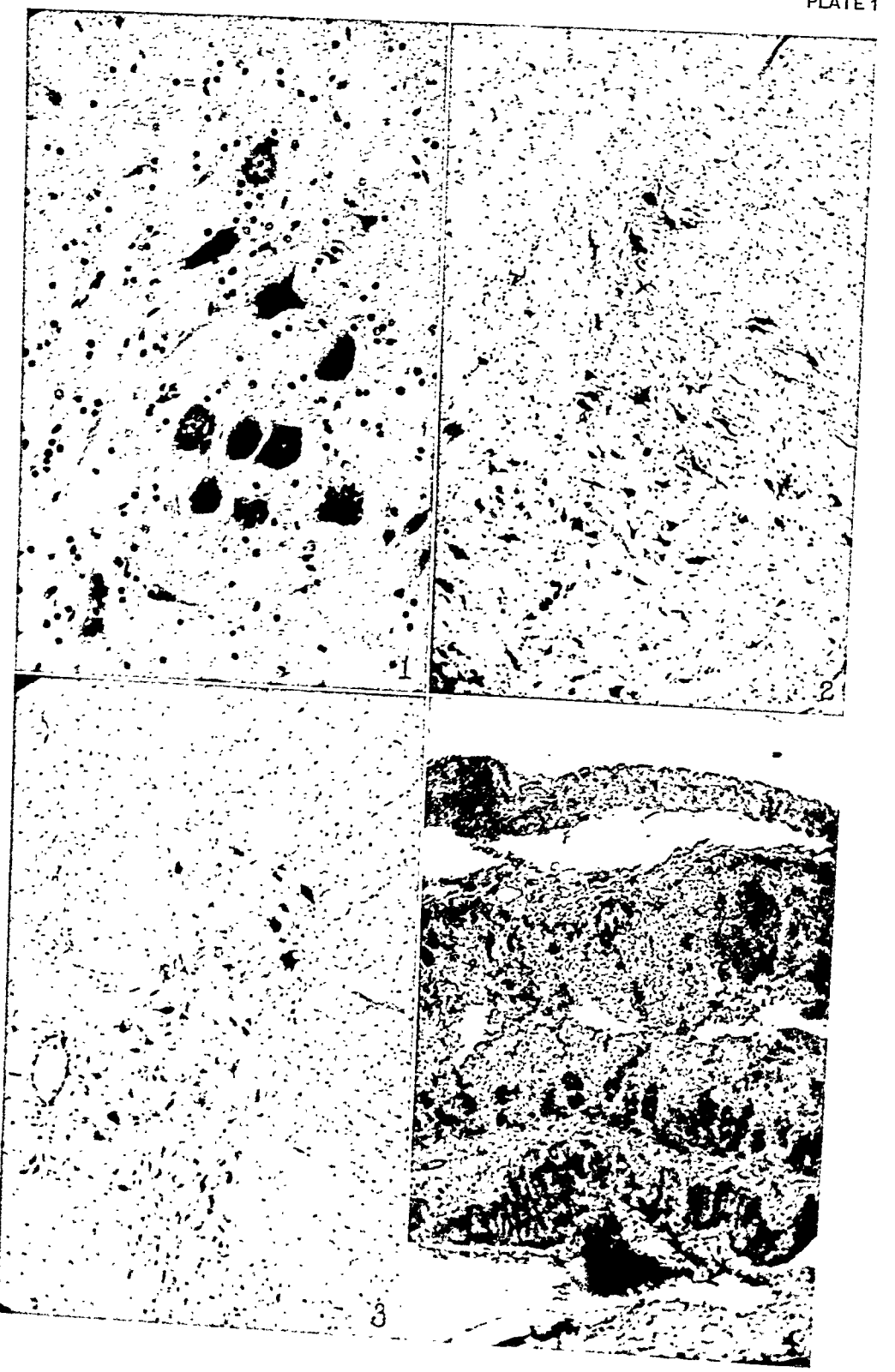
PLATE 2

FIG. 5. Rabbit 1-38 injected intravenously with 0.002 cc. S endotoxin (not heated). Diarrhea not observed, but paralysis of the extremities. Died after 60 hours. The section from cecum demonstrates hemorrhages and hyperemia in the mucosa, necrosis of the superficial cells. Considerable edema in the submucosa.

FIG. 6. Rabbit 2-87 injected intravenously with 4 cc. R exotoxin. Died after 20 hours. The section from the heart shows the marked hyperemia in the muscular wall.

FIG. 7. Rabbit 1-35 injected intravenously with 1 cc. R endotoxin (not heated). Died after 12 hours. The section from the liver demonstrates that some of the cells (the peripheral) are lighter colored than the others.

FIG. 8. Rabbit 1-35. The section from the kidney shows the peculiar appearance of the cells in tubuli contorti. In most of the cells the protoplasm is unstained.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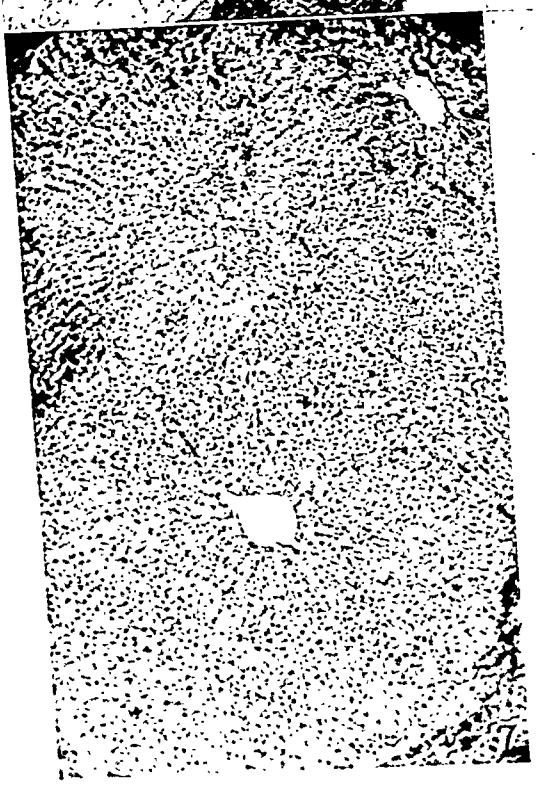

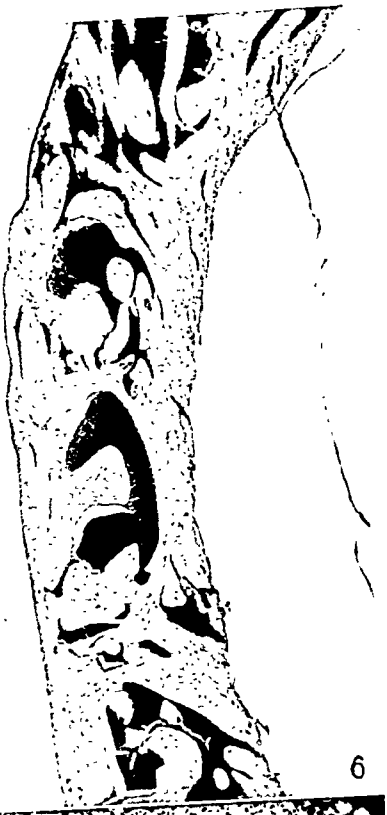

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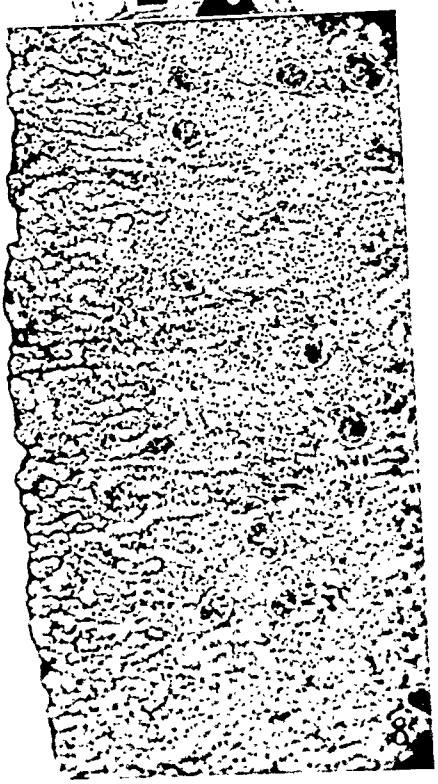

(Wasker: Tissue preparation of Silver Lines)

PATHOLOGY OF PNEUMOCOCCUS INFECTION IN MICE FOLLOWING INTRANASAL INSTILLATION

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PLATES 3 TO 5

(Received for publication, October 8, 1935)

Webster and Clow (1) have shown that mice raised under controlled conditions present marked individual responses to pneumococcus infection; for example, a complete refractory state, a prolonged carrier condition, cervical adenitis with or without fatal termination, fatal pneumonia, or death from septicemia without any localization. Moreover, Webster had pointed out in an earlier paper (2) that individual breeds of mice behave in a predictable way and differently towards intranasal infection.

A study of the pathological changes in these mice has seemed warranted in order to determine not only the individual differences in mice belonging to one breed, but also the differences in the picture produced in the different breeds by the same strain of pneumococci and the reaction of identical groups of mice all of one breed to infection with pneumococci belonging to different types. The work to be presented shows that, contrary to previous reports, provided strains of pneumococci be selected on the basis of their intranasal as distinct from their intraperitoneal virulence, and the balance of virulence of organisms on the one hand and resistance of the mice on the other be so chosen that the mice succumb in not less than 4 days, a pneumonia can be evoked in mice which have received no preparatory treatment.

In 1924 Stillman and Branch reported the production of pneumococcus pneumonia in alcoholized mice (3) and gave a description of the lesions produced (4). The percentage of mice showing evidences of localization in the lung in the groups previously immunized with homologous or even heterologous pneumococci was far greater than in the non-immunized groups. Congestion of the interalveolar capillaries was the first lesion they noted, and this was often associated with a serofibrinous pleurisy without any other lesion in the lung. The congestion was

followed by interstitial inflammation of the alveolar walls and dilatation of the perivascular lymphatics which contained cell debris; later, the alveoli contained red cells and polymorphonuclears with a little fibrin. The most advanced stage showed a leucocytic exudate and extreme anemia of the alveolar walls. The initial lesion was in the alveoli and spread occurred both centripetally and centrifugally.

Stillman and Branch studied the inception of the lesion (5) and noted, 6 hours after spraying the animal with pneumococci, small areas of interstitial inflammation of the alveolar wall with a very slight amount of exudate.

Griffith (6) twice noted consolidation of the lungs and bilateral pleural effusion in mice dying with Type II pneumococcus infection after having been immunized beforehand with Type I serum, and also a grey consolidation of one lobe in a mouse succumbing to a Type I pneumococcus of low invasiveness. He gives no account of a histological study.

More recently, Neufeld and Kuhn (7) have described pneumonia in etherized mice infected intranasally with Type I and Type XIX strains of pneumococcus. Unless the mice were anesthetized when the inoculation was made, pneumonia did not develop, save in two cases in which the mice had been infected intranasally daily for 2 weeks with a Type XIX strain. The histological picture of the Type XIX pneumonia, briefly described, is said to be similar to the picture in experimental pneumonia in the monkey (8) and in human lobar pneumonia. Hoyle (9) also reports the production of all stages from mild congestion to severe pneumonia in etherized mice infected intranasally with pneumococci.

Little has been published on pathological changes in the other organs of mice following pneumococcus infections. As stated above, Webster and Clow (1) noted cervical adenitis, and Neufeld and Etinger-Tulczynska (10) also noted swelling of the whole face in mice infected with Type I organisms, a condition which, as will be pointed out below, is probably secondary to a cervical adenitis.

Material and Technique

For our experiments several inbred breeds of mice and many different strains and types of pneumococci were used. All strains of pneumococci used were freshly isolated from human cases of pneumonia. It is important to note at this point that, as Griffith (6) and Webster and Clow (1) have shown, not all strains are suitable for certain phases of the work, especially for those having to do with intranasal virulence and the production of pneumonia. A strain may be of maximum intraperitoneal virulence and yet fail to produce death or disease even when introduced into the noses of highly susceptible mice. The converse, high intranasal and low intraperitoneal virulence, is also found though the intraperitoneal virulence can never be very low. Thus, strains of moderate intraperitoneal virulence may have a high intranasal virulence, whereas strains of very low intraperitoneal virulence do not kill when introduced into the nose. The use of inbred strains of mice which will react in a predictable manner and of

carefully selected strains of pneumococci has proved of the utmost importance. Neglect of these two essentials will explain many of the inconsistencies and failures of the past. Observance of them provides the delicate balance between organism and host, which Wadsworth (11) deems essential to the consistent production of pneumonia. It is often necessary to test as many as 50 or 60 strains of pneumococci to obtain one or two with an appreciable intranasal virulence even for highly susceptible mice, and this is especially true when Type III strains, which are most frequently intranasally virulent, are excluded.

In the case of each breed it was possible, after a few preliminary experiments, to predict within certain limits the mortality rate and the incubation time following the intranasal inoculation of a given strain of pneumococci. The technique of inoculation has already been described (1). The inoculated mice were placed in separate cages and kept under observation for the next 2 or 3 weeks. Whenever possible, a postmortem examination was performed immediately on death and animals in a moribund condition were sacrificed for this purpose, though no mice were killed until there was a certainty that they would die shortly. In some cases, death occurred during the night and postmortem changes had set in when the autopsy was performed. A note was always made to this effect. Cultures of the heart's blood were taken at autopsy. All gross lesions were noted, and besides the lungs, the kidneys, liver, spleen, and cervical lymph nodes were taken for section and fixed in Zenker's fluid. A tube was passed down the trachea, the lungs filled with fixative under gentle pressure, the tube withdrawn, the trachea tied off, and the lungs fixed *in toto* before removal. Blocks of both were so prepared as to yield sections containing a portion of each lobe. In this way, a representative picture was obtained of both lungs. In a few cases serial sections were made. Sections were stained in eosin-methylene blue or hematoxylin and eosin.

While chief attention has been paid to the pneumonia and its development, the lesions in the other organs have been studied as well. The general pathological picture will be described first, both in relation to the different breeds of mice and to the different types of pneumococci.

Lesions in Different Breeds of Mice

Numerous scattered observations in this laboratory had shown that the lesions produced in different breeds of mice following the intranasal inoculation of one and the same strain of pneumococci differed from breed to breed. Experiments were undertaken to bring out this point.

Twenty albino resistant mice and twenty albino susceptible mice, both of Institute stocks, were each given an intranasal inoculation of 0.02 cc. of a 1/100

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dilution of an 18 hour culture of Type III pneumococcus obtained from the heart's blood of a mouse dying of an intranasal inoculation with this organism.

The mortality was high in the susceptible group—65 per cent—, and low in the resistant group,—25 per cent. The susceptibles, moreover, were the first to succumb, and for those that died in this group the average survival time was only 72.5 hours compared with 120.8 hours in the resistants.

An examination of the sections prepared from the different organs revealed the fact that there was a difference in the manner in which the two breeds of mice reacted to the intranasal inoculation of the same strain of microorganism. The circumstances under which the test was made give assurance that the only variable factor lay in the breed of mice inoculated.

Lungs.—The lungs from the two groups showed quantitative differences. Macroscopically, the resistant mice showed no changes while three of the susceptible group showed consolidation and one showed serofibrinous pleurisy. In the resistant group, despite the fact that survival time was longer, all five mice showed very mild lesions microscopically. All showed congestion and early dilatation of the perivascular lymphatics, in four cases the channels containing only fluid and a few organisms. In the fifth, there were also polymorphonuclears and debris. One case showed very early infiltration of the alveolar walls in a small area and another a little fluid exudate into the alveoli.

In the susceptible group the changes were definitely more marked, but in eleven out of the thirteen cases could still be described as mild. There were congestion and perivascular lymphatic involvement, with mononuclear cells in the dilated lymph vessels. Three cases showed moderately large areas of exudate both interstitially in the alveolar walls where it consisted of leucocytes, and in the alveoli, consisting chiefly of fluid and red cells with a few polymorphonuclears. In one mouse which died in 75 hours the changes were similar in nature, but considerably more advanced.

Cervical Nodes.—The changes in the lymphatic system were next in importance after those in the lung. The lymph nodes from parts of the body other than the cervical region, except those at the hila of the lungs and occasionally those of the pre-aortic group, showed no changes. A pelvic node was frequently included in the section of the kidney, and was invariably normal. In the resistant group the changes in the cervical nodes were always conspicuous. The nodes,—which in the gross were large and opaque,—proved microscopically to be the site of large abscesses which completely or almost completely replaced the normal structure. This lesion was present in every mouse. In the susceptible group nine of the thirteen showed changes. Macroscopically, the nodes appeared

enlarged, but in only one case were they opaque. In four, the microscopic lesions were mild and consisted only of a dilatation of the peripheral sinuses which contained numbers of mononuclear cells and a few organisms. In five cases there were abscesses similar to those found in the resistant group.

Spleen.—The striking picture in the spleens of the resistant group is the necrosis or abscess formation in the follicles. In four of the five mice there was abscess formation in most of the follicles. In the susceptible group the lesion was less advanced than in the resistant group. In ten of the thirteen there were changes in the follicles, and in seven of these the lesion amounted to abscess formation. One spleen was normal and the other two showed an increased number of mononuclear cells in the pulp.

Kidney.—Normal in both groups.

Liver.—Few lesions in any of the mice. However, two resistant mice showed thromboses of the branches of the portal vein with infarcts of the liver.

An attempt was made to repeat this study of the lesions in different breeds of mice with other than Type III pneumococci. The difficulty encountered was to find a strain which would kill resistant albino mice when inoculated intranasally. One intranasally virulent strain was obtained, however.

An 18 hour culture of a Type XIX strain, which had proved highly virulent for Swiss mice when inoculated intranasally, was given intranasally to 20 resistant albinos and 20 susceptible albinos. The technique followed was that already given. The mortality rate and average survival time of the susceptible group was 55 per cent and 77.7 hours. In the resistant group only three died (mortality 15 per cent), and all of these died early (survival time 52.3 hours).

In the resistant group there was no evidence of localization in the lungs. One mouse showed a bilateral serofibrinous pleurisy, but there were no demonstrable lesions in the underlying lung. The other two showed only capillary congestion. In two of the three there were slight changes in the kidneys—granular debris in Bowman's capsule and convoluted tubules and some dilatation of the convoluted tubules. The other organs were normal.

In the susceptible group eight of the eleven mice showed some evidence of localization in the lungs. Of these, four had shown consolidation recognizable macroscopically. In three there was only capillary congestion. Four of the other eight showed only slight interstitial accumulation of polymorphonuclears and monocytes with dilatation of the perivascular lymphatics which contained fluid and a few cells. In the other four mice the lesions were advanced. They consisted of interstitial accumulation of cells and fluid in the alveolar septae, exudate of fluid with some red cells and polymorphonuclears into the alveoli, which was in some instances lobar in extent, some dilatation of the perivascular lymphatics which contained fluid and some cells, and in two cases a bilateral serofibrinous pleural exudate. In the other organs the changes were slight, save in

the kidney. Two of the eleven mice showed a frank diffuse nephritis with fibrin thrombi in the glomerular loops, changes in the cells of the convoluted tubules which contained colloid droplets or were frankly necrotic, dilatation of these tubules, and granular casts. In four others there were moderate tubular changes. In one case, the liver showed many areas of necrosis, and in two there were very small areas of necrosis in the splenic pulp.

In summary, of the lesions in the two breeds of mice, it can be said that there were definite quantitative differences in the two breeds (Table I). Thus, in the experiment with Type III organisms, the resistant mice showed very slight changes in the lungs, but marked lesions in the cervical nodes and follicles of the spleen. The susceptible mice showed more advanced lesions in the lungs, but less marked

TABLE I

Breed of mice	Type of pneumococcus	Lesion in organs		
		Lungs	Lymphatic system	Kidneys
Resistant	III	± (interstitial)	++	0
Susceptible	III	++ (interstitial)	+	0
Resistant	XIX	0	0	±
Susceptible	XIX	++ (interstitial)	0	+ (glomerular)
Unselected	I	++ (diffuse)	±	++ (glomerular)
Unselected	II	++ (confluent)	±	+ (tubular)
Unselected	III	++ (interstitial)	++	0

changes in the lymphoid tissue of the cervical nodes and spleen. In the experiments with Type XIX organisms the differences in the lungs were even more striking, although the small number of resistant mice which succumbed might raise the question as to the significance of the differences in this one particular case. Thus, none of the three resistant mice showed any local reaction in the lung, though one showed serofibrinous pleurisy. On the other hand, eight of the eleven susceptible mice showed local reaction in the lung, and in four of these the changes were advanced. It was impossible to be certain of differences in other organs, but it was possible that the susceptible group showed a higher incidence of nephritis and of significant changes in the liver.

Lesions Produced by Different Types of Pneumococci

Although different strains of the same type of pneumococcus differed in virulence when introduced intranasally into a standard group of mice, nevertheless the end result in those mice which died was found to be the same. Thus, it was found that, despite the varying mortality rate caused by different strains of the same type of pneumococcus, the lesions produced in those mice that died were closely similar for the one type of pneumococcus in a given breed of mice and were predictable. They differed, however, with the type of organism used. There had been few observations on this latter point.

The variation with the type of organism used is clearly brought out by the pathological changes found in those unselected albino mice which died as a result of the intranasal inoculation of different types of pneumococcus, as described elsewhere (1). Autopsies were performed on mice dying from the intranasal inoculation of Types I, II, III, and V strains. Three strains of Type I were used, four of Type II, two of Type III, and one of Type V. Twelve mice died from both Type I and Type II strains, eleven from Type III strains, and only one from the Type V strain. In every case in this experiment an autopsy was performed within 30 minutes of the time of death of the mouse, and no mice dying during the night were included. Since the lesions were very similar for the strains of one type but differed from type to type, the mice dying from infection with each type will be described together.

Type I and Type V.—The lesions in the single mouse dying from a Type V infection were so similar to those of the Type I group that they will be described with this group.

Macroscopically, the changes in the thoracic cavity were the most conspicuous. Pneumonic consolidation was infrequent, but the lungs were usually dark red and moist on section, unlike the normal pink, crepitant lung. Pleurisy was common and consisted usually of a thin layer of fibrin dulling the shiny surface of the lung. Effusion when present was scanty, thin, and watery. The bronchial and paratracheal nodes were normal. The cervical nodes were occasionally enlarged and opaque, but this was not a common finding. The spleen was also enlarged in several instances, but without macroscopic changes. In a certain number of cases petechiae could be seen on the surface of the kidneys.

Microscopically, one mouse out of the thirteen showed completely normal lungs, and in two others the lesions were very slight. The rest showed moderate or marked changes. In what appeared to be the earliest stages, the interalveolar

capillaries were dilated and filled with blood. There was often a slight pleural exudate of fibrin and polymorphonuclears, and the subjacent lymphatics would be filled with an exudate similar to that on the pleura. The blood vessels showed a dilatation of spaces in the region of, or more probably just outside, the adventitia which were presumably lymphatics. This dilatation increased in extent as the vessels ran centripetally, and the spaces contained serous fluid, fibrin, and pneumococci with but few cells. In this early stage there was very little exudate into the alveoli, and what was present consisted of fluid and pneumococci usually with a very few cells. The changes were scattered diffusely through both lungs. The blood vessels contained many pneumococci. In the later stages all changes became markedly increased. The vascular engorgement was extreme and the alveolar capillaries contained large numbers of leucocytes, both polymorphonuclears and monocytes. Pleural exudate when present was conspicuous and consisted of a layer, 1 mm. or more in thickness, of fibrin, polymorphonuclears, and organisms. The subpleural lymphatics were sharply outlined with their content of polymorphonuclears, fluid, and organisms, and this could be followed through the perivascular lymphatics to the hilum. The peribronchial lymphatics were very rarely involved. There was more exudate into the alveoli. In some places this was diffuse and consisted of serous fluid and pneumococci with relatively few cells, while in others it was found in small localized areas of a few alveoli and consisted of masses of leucocytes and a little fibrin tightly interwoven (Figs. 1, 2, 3). Pneumococci were not as plentiful in the blood stream as in the earlier stages.

The cervical nodes were enlarged, but rarely showed definite lesions. Occasionally there was an acute lymphangitis of the entering or leaving lymphatics which were dilated and filled with fluid, polymorphonuclears, and pneumococci. These nodes lie in close relation to the salivary glands and presumably drain the mucosa of the nasopharynx and throat. The spleen was also enlarged and occasionally showed necrosis of the Malpighian corpuscles. The normal structure was lost and the corpuscles were occupied by masses of polymorphonuclears, debris, and pneumococci. In one case there were several small infarcts in the liver, but otherwise this organ showed little but cloudy swelling or fat in the cells at the periphery of the lobule. The kidneys were the seat of a marked change which amounted in many cases to a diffuse nephritis. There was some proliferation of the endothelial cells of the glomerular capillaries, and in many cases the latter were occluded with fibrin thrombi (Fig. 12). The glomerular capsule spaces contained debris, and adhesions between tuft and capsule wall were found. In a few cases blood was found in the tubules that had come presumably from the glomerulus above. Besides blood, the dilated tubules contained loose casts of debris and, occasionally, pneumococci. The cells of the convoluted tubules frequently contained small hyaline droplets, described elsewhere (12) as colloid granules, and in a certain number of cases this lesion was striking. In all, ten out of the twelve mice showed changes which could be described as acute diffuse nephritis.

Type II.—Macroscopically, the most conspicuous changes were again in the thoracic cavity. Consolidation, which was often lobar in extent, was more commonly observed than in the other series, while the dark red, moist lung and the rather dry pleurisy were similar to those changes noted in the Type I group. It was very unusual to find any change in the lymph nodes whether tracheal, bronchial, or cervical, though in one case the latter were slightly enlarged and opaque. The spleen was not infrequently several times its normal size but showed no macroscopic lesions. The kidneys and other organs showed no changes.

Microscopically, in the ten of the twelve mice which showed lesions in the lung the peculiarity lay in the greater amount of exudate into the alveoli in the Type II infection than in those due to Type I or Type III infections. Even in the early stages the exudate was conspicuous, the alveoli containing large amounts of serous fluid and many pneumococci, together with a few cells of which the larger number were red cells. Other changes included capillary dilatation; early pleural effusion and exudate; dilatation of the subpleural lymphatics which were filled with fluid, cells, and organisms; and many organisms in the blood vessels. In the later stages, the alveolar exudate showed an increase in the number of leucocytes, and a slight deposition of fibrin (Figs. 4, 5). The organisms were present in the alveolar exudate but had decreased in the blood. The pleural exudate and involvement of the perivascular lymphatics were more marked.

No changes were noted in the cervical nodes. In the spleen the follicles occasionally showed areas of necrosis, at times so extensive as to obliterate the whole follicle. In one case, the portal veins of the liver contained thrombi and these had produced small cortical infarcts. The changes in the kidney were not as striking as with the Type I infection. The glomerular capsular spaces were dilated and contained albuminous fluid and pink hyaline debris. In two cases, the convoluted tubules contained small amounts of blood coming presumably from the glomerular tuft above. The most conspicuous changes were in the tubules. These were dilated, and the lining cells showed cloudy swelling and occasional necrosis with a loss of cytoplasm into the lumen of the tubule; special stains revealed colloid granules in the cytoplasm in many cases (Fig. 13). The tubules contained well formed casts of loose plugs of debris, the latter coming both from the glomerular transudate and from the cytoplasm of the cells of the tubules.

Type III.—Macroscopically, the picture of the infection due to Type III strains differed from those already described. The changes in the thoracic cavity were still very striking, but not in every case were they the most conspicuous lesion. Pleurisy with a copious gelatinous exudate was a very frequent finding. It was often associated with a mediastinitis and pericarditis, less frequently with a peritonitis. The change most commonly seen in the lungs was a large, moist, dark red lung; but consolidation, which tended to be lobular, was more common than in Type I cases. The other conspicuous change was in the lymphoid tissue. Bronchial and tracheal nodes were sometimes enlarged and opaque or even frankly

necrotic, but most striking were the changes in the cervical nodes which were usually enlarged and opaque, often necrotic and occasionally, the infection having spread to the surrounding tissues, were actually sloughing through the skin. In many cases the swollen lymph nodes obstructed the free return of lymph and the face region of the affected mice became swollen and edematous, especially on the side on which the nodes were principally affected (Fig. 15). The spleen was constantly enlarged, filled with blood, and showed opaque yellow nodules occupying the lymph follicles. In three cases, the enormously engorged spleen had ruptured and an extensive hemorrhage had occurred into the peritoneum. The other organs showed no macroscopic changes.

Microscopically, two of the mice showed no lesions in the lungs. In the other nine, the lesions differed from those due to either Type I or Type II strains. A most important feature was the tendency for the greater part of the lesion to be interstitial and localized in the alveolar septum. This, in the earliest stages, was shown by the very marked dilatation and engorgement of the alveolar capillaries (Fig. 6). At first, leucocytes were scanty, but later they increased in number. Diapedesis took place and large numbers of leucocytes together with fluid and some fibrin collected outside the vessels but within the septa (Figs. 7, 10). Later, there was a true interstitial inflammation progressing as far as necrosis of the septa. In two cases of the Type III infection, infiltration occurred round the main bronchi, a lesion never observed with the other types. The peribronchial lymph spaces were dilated and contained fluid, leucocytes, and a small amount of fibrin. Subpleural and perivascular lymphatic infiltration, similar to that described above, was also present. Alveolar exudate was similar to that found in Type I. In one case there was a plug of cells and debris in the bronchus, but in all cases the bronchial mucosa was intact.

Lesions were found in the lymph nodes at the hilum of the lung, in the cervical nodes, and in the follicles of the spleen. In the nodes, the change began as an enlargement of the node due to a swelling of the peripheral lymphatics which were filled with mononuclear cells and some organisms. Later, polymorphonuclears appeared and increased in number, while at the same time the pneumococci became more plentiful and areas of necrosis appeared. These increased in size until they filled the whole node, the normal structure of which was completely destroyed (Fig. 14). There could be found an acute lymphangitis of the entering or leaving lymphatics. The lesions in the cervical nodes were the most conspicuous and were often present when the nodes at the hilum of the lung were normal. In the follicles of the spleen, the lesion began in the center. Some of the lymphoid cells appeared necrotic and pneumococci could be seen in the neighborhood. Following this, there was a loss of most of the lymphoid cells, though whether chiefly from necrosis or from migration away from the site could not be determined, and only the framework of the follicle remained with polymorphonuclears lying at the periphery. Still later, these polymorphonuclears filled the follicle round a necrotic core, and a true abscess was formed (Fig. 16). In one case, small areas of necrosis were found in the liver, but otherwise there was nothing but cloudy swelling. The kidney showed nothing but cloudy swelling of the epithelium of the tubules.

In summarizing the lesions produced in a standard breed of mice by strains of pneumococci belonging to different types, the following points are most worthy of emphasis (Table I). As far as the lungs are concerned, the lesions differed considerably in the three types. In Type I, the changes were diffuse, without any particular point of localization and without lobar distribution. In the lungs from the Type II infections, the exudate into the alveoli and the lobar distribution were the most conspicuous features, while pleural exudate was infrequent. In the Type III cases, the localization of the lesion in the interstitial tissue of the alveolar septa was the most conspicuous difference, but there was also to be observed a very infrequent peribronchial infiltration. Copious gelatinous pleural exudate was also a feature of the Type III infections. In this group also were found the marked lesions in the lymphatic system, cervical nodes, hilar nodes, and lymph follicles of the spleen. Similar changes were found in the mice infected with the other types, but much more rarely and in lesser degree.

In the other organs the renal lesions were most striking. In ten out of twelve Type I cases there was an acute diffuse nephritis; in the Type II cases lesions were also found, but were predominantly tubular; while the kidneys of the Type III infections showed no lesions worthy of note. It is interesting that rabbits infected with Type I, Type II, and Type III strains of pneumococci showed renal lesions only with the Type I strains (12, 13), thus resembling the mice, but it must be stated that in the rabbits no attention was paid to the virulence of the strains of pneumococci used, and that most of them were stock strains.

The Development of the Pneumonia Following Intranasal Inoculation

In order to obtain information as to the origin and mode of spread of this pneumonia in mice following intranasal inoculation, serial sections of lungs were examined. It is realized, of course, that this method can merely suggest the point of origin and method of spread, and shows little or nothing as to the method by which the organisms reached the presumed point of origin. Serial sections were made only in Type III pneumonias.

As a result of the examination and a comparison of these findings with those obtained by the routine examination of several sections

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As a result of the examination and a comparison of these findings with those obtained by the routine examination of several sections

from each lung, certain statements can be made. The earliest lesion in every case of Type III pneumonia, and the only one which is found by itself, is a dilatation and engorgement of the capillaries, especially those of the interalveolar septa (Fig. 6).

It is difficult to say which, if any, of the three changes next to be described precedes the others. These are: the collection of cells and fluid in the alveolar septa but outside the capillaries—an interstitial lesion (Figs. 7, 10); the exudate of albuminous fluid, rich in organisms but poor in cells, into the alveoli (Figs. 8, 11); and the dilatation of the subpleural and perivascular (but not the peribronchial) lymphatics with fluid, pneumococci, and monocytic cells.

The first two changes—the early exudate and the interalveolar inflammation—occur apparently independently in different parts of the lung or different parts of the same lobe. In a certain number of cases the interstitial lesion seems to precede the exudate and is well marked in the walls of alveoli in which exudate is just appearing. Yet, this is not always so, and often the walls of alveoli filled with exudate appear merely congested. It may be that in such cases the interstitial inflammation has been of ephemeral character and has already disappeared. The lymphatic involvement seems to follow a little later, and this is probably always the case with the subpleural lymphatics. It may appear in some microscopical preparations that lesions are present in the perivascular lymphatics in lobes with no other lesion save engorgement, but it has never proved possible to demonstrate this in serial section.

One can state then that in the mouse the pulmonary changes seem to progress in the following stages: (*a*) engorgement; (*b*) interalveolar interstitial exudate; (*c*) an albuminous fluid exudate into the alveoli and into the perivascular and subpleural lymphatics draining the affected region.

From now onwards, each separate process develops almost independently. The alveolar exudate becomes more cellular and eventually strands of fibrin, always less in amount than in pneumonia in man, are laid down. The interstitial inflammation may subside, but occasionally becomes so intense that the whole septum is destroyed and its structure lost. The subpleural and perivascular lymphatics become still more dilated and the contents become largely cellular

with polymorphonuclears predominating (Fig. 9). The pleura overlying the affected lymphatics is the site of an inflammatory exudate—an acute pleurisy—and this may be almost as early a lesion as the exudate into the alveoli.

The spread along the lymphatics can be followed to the nodes at the hilum. In these, the earliest change is one of dilated sinusoids which contain mononuclear cells and a few organisms. Later, these nodes become the seat of an intense inflammation so that the whole organ is converted into an abscess which ruptures and the organisms escape out into the mediastinum to produce an acute mediastinitis. The paratracheal nodes, lying higher up along the trachea, are always affected much later.

The cervical adenitis appears to have no direct connection with the pulmonary lesions as outlined here. In many cases—perhaps the majority—the most severe cervical adenitis is found in association with lungs that are almost normal or show little else than congestion.

SUMMARY

Pneumonia can be produced in mice, which have not been previously prepared, by intranasal inoculation of broth cultures of certain strains of pneumococci.

Lesions which are quantitatively different can be produced in different breeds of mice by inoculation of the same type of pneumococcus. Similar inoculation of different types of pneumococci into one breed of mice results in lesions which are qualitatively different.

In general, these lesions are as follows: a diffuse pneumonia and an acute glomerular nephritis in unselected mice receiving Type I strains; a confluent pneumonia and a tubular nephritis in the case of Type II strains; and as result of Type III strains, an interstitial pneumonia with extensive gelatinous pleurisy, together with necrosis and abscess formation in the spleen and cervical lymph nodes. Resistant strains of mice with Type III pneumococci show slight changes in the lungs, but marked lesions in the spleen and cervical nodes, while susceptible mice with the same type of pneumococcus show marked changes in the lung and moderate lesions in the spleen and cervical nodes.

The method of development of Type III pneumonia, studied by means of serial sections of nasally infected mice, appears to proceed

in the stages of vascular engorgement, interalveolar interstitial exudate, albuminous fluid exudate into the alveoli and the perivascular lymphatics draining the affected site, and finally, a frank pneumonia with a cellular exudate in the alveoli but without much fibrin.

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EXPLANATION OF PLATES

PLATE 3

FIG. 1. Type I pneumonia. Localized area of leucocytic exudate into alveoli just below pleura. There is a thin film of pleural exudate on the surface. Eosin-methylene blue. ×100.

FIG. 2. Type I pneumonia. Similar localized pneumonic area in the substance of the lung. At the lower right corner a portion of a blood vessel appears with a dilated lymphatic channel surrounding it, which is filled with leucocytes. Eosin-methylene blue. ×100.

FIG. 3. Type I pneumonia. Higher magnification showing the type of cells forming the alveolar exudate. Eosin-methylene blue. ×650.

FIG. 4. Type II pneumonia. Confluent area of pneumonia. The alveoli contain masses of leucocytes and necrotic debris. The lymphatic channels around the two larger vessels are greatly dilated and filled with leucocytes and debris. Eosin-methylene blue. ×100.

FIG. 5. Type II pneumonia. Higher magnification showing the alveolar exudate of leucocytes and debris. Eosin-methylene blue. ×650.

PLATE 4

FIG. 6. Type III pneumonia. Showing the general vascular engorgement. Eosin-methylene blue. ×100.

FIG. 7. Type III pneumonia. Great thickening of the alveolar walls with the collection of leucocytes inside and outside the alveolar capillaries. Eosin-methylene blue. ×100.

FIG. 8. Type III pneumonia. Showing many alveoli filled with albuminous fluid. Eosin-methylene blue. ×100.

FIG. 9. Type III pneumonia. Most of the alveoli are filled with a mass of leucocytes, debris, and fibrin. Many alveolar walls are necrotic. In the lower part of the field is a blood vessel with an enormously distended lymphatic channel filled with fibrin and leucocytes. Hematoxylin-eosin. ×100.

FIG. 10. Type III pneumonia. High magnification showing the interstitial infiltration of the alveolar walls. Eosin-methylene blue. ×650.

FIG. 11. Type III pneumonia. High magnification showing the alveoli filled with albuminous fluid and a few cells. There is interstitial infiltration of the walls. Eosin-methylene blue. ×650.

PLATE 5

FIG. 12. Type I infection. Renal glomerulus with capillary loops occluded with fibrin thrombi. Weigert's fibrin stain. ×650.

FIG. 13. Type II infection. Deeply staining "colloid" granules in the cells of the convoluted tubules. Weigert's fibrin stain. ×650.

FIG. 14. Type III infection. Cervical lymph node the center of which is occupied by an abscess. The main lymph vessel to the right also shows inflammation. Portions of the salivary glands may be seen. Eosin-methylene blue. ×100.

FIG. 15. Type III infection. Albino mouse showing ulceration through the skin below the left mandible (three dark areas through the hair), and great swelling of the whole head and face from edema. The eye is closed by the edema. Natural size.

FIG. 16. Type III infection. Spleen showing outline of Malpighian corpuscle the center of which is occupied by an abscess. Eosin-methylene blue. ×100.

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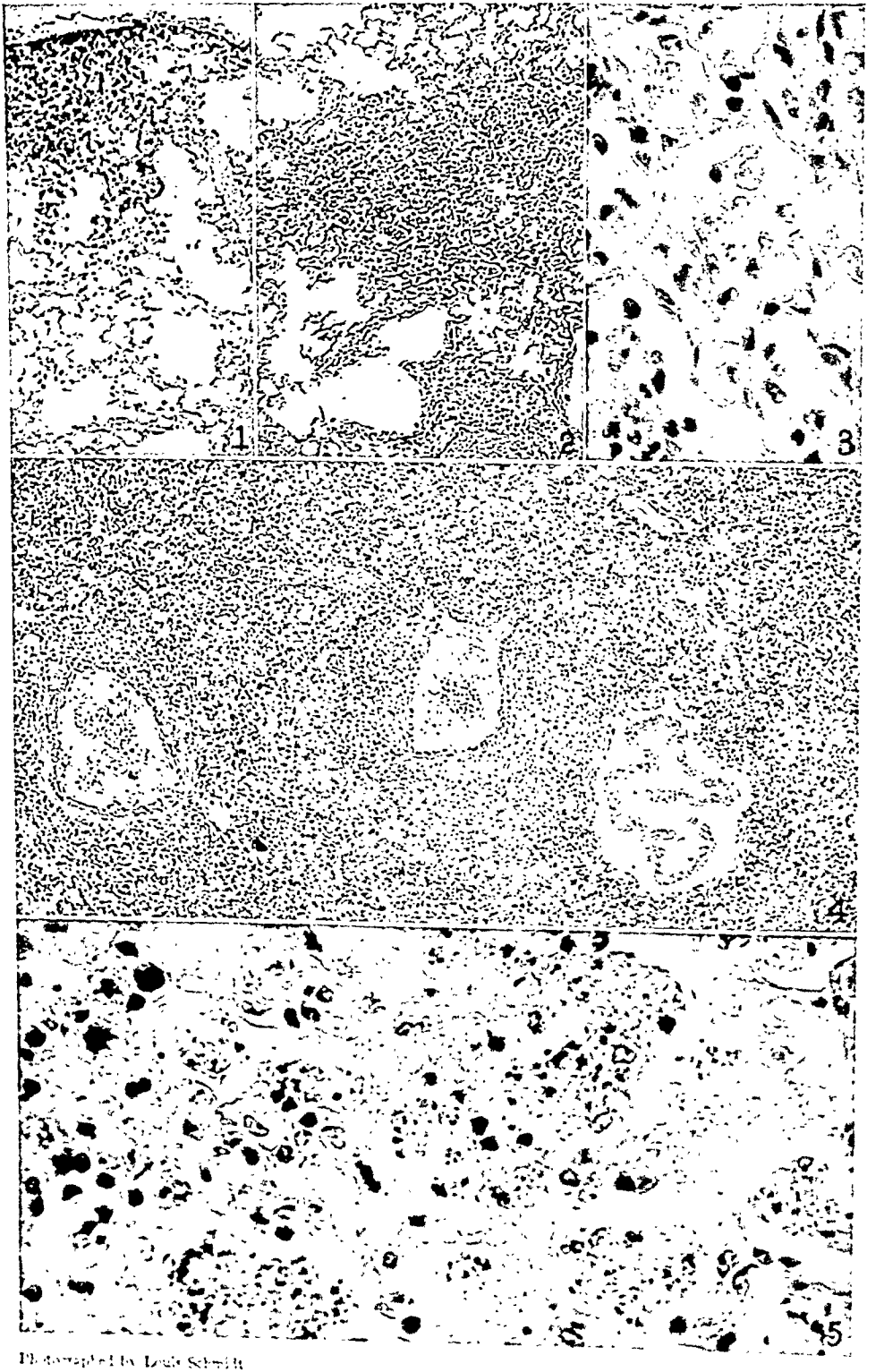
PLATE 4

FIG. 6. Type III pneumonia. Showing the general vascular engorgement. Eosin-methylene blue. ×100.



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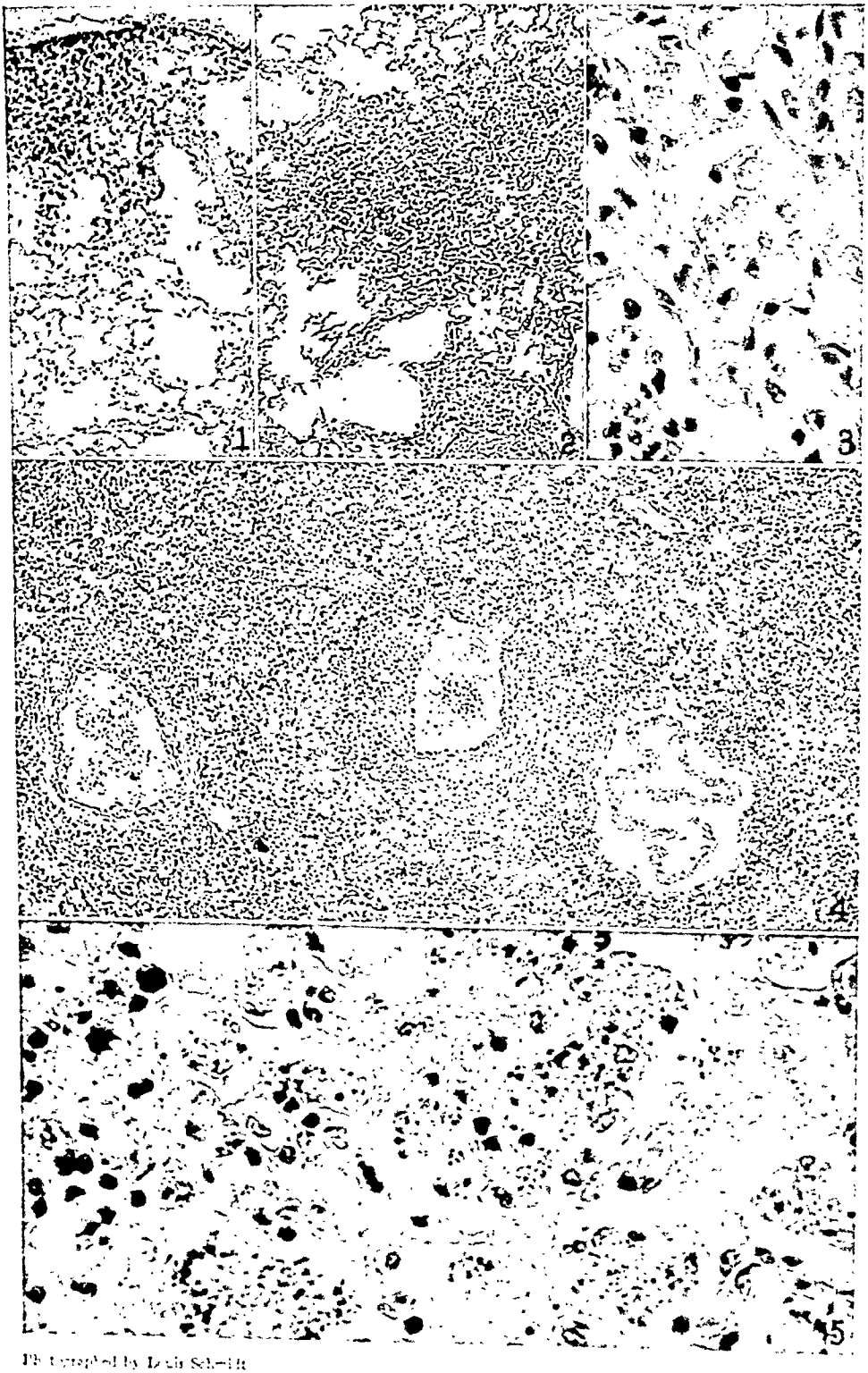

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(Rake Pneumococcus infection in mice)

(Kelly, Lange & Associates)

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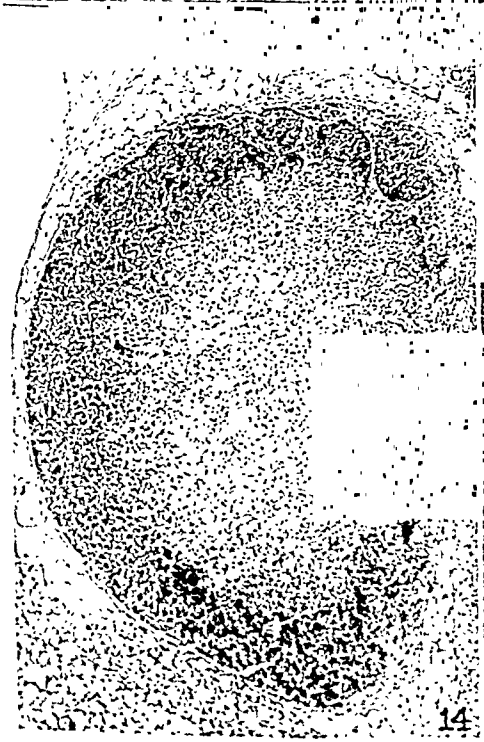

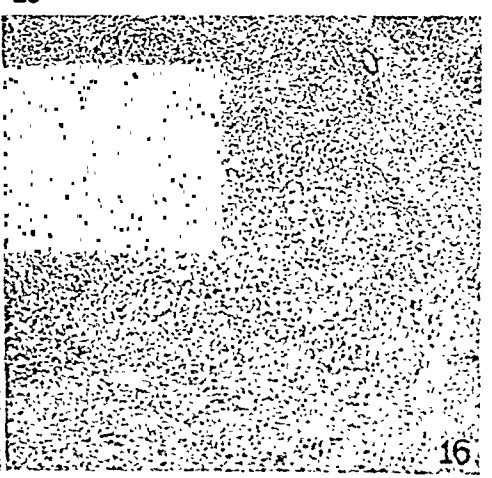

Photographed by Louis Schmidt

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INFECTIOUS FIBROMA OF RABBITS

III. The Serial Transmission of Virus myxomatosum in Cottontail Rabbits, and Cross-Immunity Tests with the Fibroma Virus

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In a previous paper (1) it was suggested, on the basis of immunological similarities between the viruses of infectious fibroma and infectious myxoma, that passage of myxoma virus through cottontail rabbits (genus *Sylvilagus*) might yield fibroma virus just as passage of variola virus through calves supposedly yields vaccinia virus. The use of cottontail rabbits to effect this hypothetical transformation was suggested by the fact that the fibroma virus was originally obtained from a naturally occurring growth in one of these animals (2).

The susceptibility of the cottontail rabbit to infectious myxoma is not established to judge from the literature on the subject. Moses (3) has stated that the wild rabbits of Brazil are insusceptible to experimental infection with *Virus myxomatosum* except in rare instances and Hobbs (4) and Hyde and Gardner (5) were unable to infect our native cottontail rabbits with it. The writer, in 3 attempts to infect cottontail rabbits by subcutaneous administration of *Virus myxomatosum*, obtained 1 doubtful infection. In this rabbit a transitory thickening of the epidermis and subcutaneous tissue developed at the site of injection 16 days after inoculation (1). It seemed likely that if any hope of establishing *Virus myxomatosum* in cottontail rabbits was to be entertained, a route of inoculation other than subcutaneous should be employed.

Attempted Infection by the Intracerebral Route

A cottontail rabbit was inoculated intracerebrally with 0.1 cc. of a dilute suspension of testicular myxoma virus. The animal exhibited no signs of illness

and was sacrificed on the 9th day. The brain, which showed no macroscopic lesions, was removed and used in preparing an approximately 10 per cent suspension. A cottontail rabbit was inoculated intracerebrally with 0.1 cc. of this suspension and in addition was injected subcutaneously with 2 cc. and intraperitoneally with 8 cc. of the suspension. A domestic rabbit was inoculated subcutaneously with 1 cc. and intratesticularly with 0.5 cc. of the suspension. The domestic rabbit died of characteristic myxoma on the 11th day, while the cottontail rabbit developed no illness and was sacrificed on the 9th day. A 10 per cent suspension of its brain was prepared and injected into a cottontail rabbit and a domestic rabbit as in the previous experiment. No evidence of myxoma appeared in the domestic rabbit and consequently no further cerebral serial passages through cottontail rabbits were attempted.

From this experiment it was apparent that *Virus myxomatosum* survived for 9 days in the brain of a cottontail rabbit and was then transmissible to a laboratory rabbit, but it probably did not increase in amount since the brain of even the second serial passage cottontail rabbit failed to infect a domestic rabbit. This route of inoculation was obviously unsatisfactory in any attempt to modify the virus by prolonged serial passage.

Infection of Cottontail Rabbits by Intratesticular Inoculation

Because of the facility with which the fibroma virus infects domestic rabbits when inoculated intratesticularly, it was decided to try this route of inoculation in infecting cottontail rabbits with *Virus myxomatosum*. It was found that regular and satisfactory infections could be obtained by testicular inoculation supplemented by simultaneous subcutaneous inoculation. In all, fifteen cottontail rabbits have been infected in this manner and two by subcutaneous inoculation alone. Most of the cottontail rabbits used in these experiments were purchased in Kansas but a few trapped in the neighborhood of the laboratory were also used. No naturally immune animals were encountered.

Course of the Disease.—The clinical picture of the disease induced in cottontail rabbits by *Virus myxomatosum* proved to be very different from that seen in domestic rabbits. The incubation period was long, varying from 6 to 12 days. The disease was an entirely local process. The first evidence of infection in all instances was a slight swelling of the inoculated testicle. Usually the subcutaneous tissue at the site of injection remained negative although rarely a small

firm tumor developed. The inoculated testicle, after swelling had begun, often increased rapidly in size and became very firm, the swelling occasionally being accompanied by edema of the scrotum. The animals, however, showed no evidence of generalized illness and in special no myxomatous swellings of the eyelids, nose, ears, or anus. None died, but 10 were sacrificed from 11 to 19 days after inoculation. The remaining 7 made uneventful recoveries, and it is believed that all 17 would have survived. The inoculated testicle frequently reached a size 2, and sometimes even 3 times that of the uninoculated testicle. This enlargement persisted for an indefinite period but in most instances retrogression had begun within 25 days following inoculation. Late in the course of the infection, when the scrotal edema had subsided, the inoculated testicle was frequently irregularly nodular.

Pathology.—The pathological picture in cottontail rabbits autopsied 11 to 19 days following infection was quite constant. Usually no lesion was present at the site of subcutaneous inoculation, though rarely a small tumor was encountered; firm, pinkish white, edematous and giving, on cut section, the impression of a fibroma. The inoculated testicle, in addition to being enlarged, was injected and varied in color from a pale pink to a deep purplish red. On cut section it was firm and moist and frequently white and fibromatous in appearance even though the surface of the testicle had appeared injected. The epididymis, sometimes relatively more enlarged than the testicle, was usually white or pinkish white in color, nodular, and cut as though fibrous. The scrotum, when involved, was thickened and its walls were diffusely infiltrated with a gelatinous exudate.

Only one subcutaneous tumor has been examined histologically. It had begun to retrogress at the time the animal bearing it was autopsied. The overlying epithelium was normal in appearance, and no cytoplasmic inclusions were observed. The main mass of the tumor had been composed of widely spaced large stellate connective tissue cells but these, at the time of examination, were degenerating and stained but faintly pink with phloxin-methylene blue. Pink-staining collagen fibrils, coagulated lymph, and many round cells filled the spaces between the degenerating connective tissue cells.

Four myxomatous cottontail rabbit testicles have been examined histologically. All presented similar pictures. There was a marked proliferation of connective tissue cells in the interstitium, and mitotic figures in some sections were plentiful. The arrangement of the cells varied; in some it was so loose and the individual cells so large and isolated that the appearance was that of a myxomatous infiltration. In other sections the cells were definitely of the young connective tissue type and formed compact whorls about the seminiferous tubules. In some portions of all sections necrotic seminiferous tubules were seen. This necrosis was probably secondary to pressure exerted by the rapidly proliferating interstitial tissue. Nests of round cells were present in all sections and, in some, large areas of the interstitium were densely infiltrated with this type of cell. No cytoplasmic inclusions were observed in epithelial cells in either the testicle or epididymis.

TABLE I

Passage of Virus myxomatosum Serially through Cottontail Rabbits

Passage No. and date	Rabbit No.	Infection with *Virus myxomatosum*		Result
		Supernatant of a 5 per cent suspension of	Dosage and route of inoculation	
1 10/31/32	DR* 4-72	Subcutaneous lesion DR 4-81 (1:50 dilution)	1 cc. s.c.† and 0.2 cc. i.t.†	Died, 11th day; typical myxoma
	CR* 4-94	Subcutaneous lesion DR 4-81	2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle greatly enlarged when killed on 17th day
2 11/17/32	DR 5-09	Testicle CR 4-94	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 10th day; typical myxoma
	CR 5-08		2 cc. s.c.	Myxomatous tumor at site of inoculation measuring 4 x 5 x 0.75 cm.; animal killed on 18th day
3 12/ 5/32	DR 5-30	Subcutaneous lesion CR 5-08	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 11th day; typical myxoma
	CR 5-32		2 cc. s.c. and 0.5 cc. i.t.	Questionable subcutaneous lesion; no general symptoms; inoculated testicle greatly enlarged when killed on 14th day
	CR 5-33		2 cc. s.c.	Moderate subcutaneous reaction by 16th day; complete and uneventful recovery
4 12/19/32	DR 5-53	Testicle CR 5-32	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 10th day; typical myxoma
	CR 5-39		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle greatly enlarged when killed on 19th day
	CR 5-42		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle greatly enlarged; allowed to recover

5 1/ 7/33	DR 5-78 CR 5-43	Testicle CR 5-39	0.5 cc. s.c. and 0.1 cc. i.t. 2 cc. s.c. and 0.5 cc. i.t.	Died, 15th day; typical but slow myxoma No subcutaneous lesion; no general symptoms; inoculated testicle moderately enlarged when killed on 16th day
6 1/23/33	DR 6-06 CR 6-02	Testicle CR 5-43	0.5 cc. s.c. and 0.1 cc. i.t. 2 cc. s.c. and 0.5 cc. i.t.	Died, 7th day; typical myxoma No subcutaneous lesion; no general symptoms; inoculated testicle greatly enlarged when killed on 14th day
	CR 6-03		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle greatly enlarged; complete and uneventful recovery
7 2/ 6/33	DR 5-93 DR 5-68 CR 5-98	Testicle CR 6-02	0.5 cc. s.c. and 0.1 cc. i.t. 0.5 cc. s.c. and 0.1 cc. i.t. 2 cc. s.c. and 0.5 cc. i.t.	Died, 12th day; typical myxoma Died, 12th day; typical myxoma No subcutaneous lesion; no general symptoms; inoculated testicle greatly enlarged when killed on 15th day
	CR 6-04		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle greatly enlarged; complete and uneventful recovery
8 2/21/33	DR 6-43 CR 6-45	Testicle CR 5-98	0.5 cc. s.c. and 0.1 cc. i.t. 2 cc. s.c. and 0.5 cc. i.t.	Killed, 10th day; typical myxoma No subcutaneous lesion; no general symptoms; inoculated testicle moderately enlarged and firm when killed on 15th day
	CR 6-34		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle moderately enlarged; complete and uneventful recovery

* DR = domestic rabbit. CR = cottontail rabbit.
† s.c. = subcutaneously. i.t. = intratesticularly into one testicle.

TABLE I—*Concluded*

Passage No. and date	Rabbit No.	Infection with *Virus myxomatosum*		Result
		Supernatant of a 5 per cent suspension of	Dosage and route of inoculation	
9 3/ 8/33	DR 6-56	Testicle CR 6-45	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 11th day; typical myxoma
	CR 5-99		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle moderately enlarged when killed on 11th day
	CR 6-32		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle moderately enlarged; complete and uneventful recovery
10 3/20/33	DR 6-64	Testicle CR 5-99	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 8th day; typical myxoma
	CR 6-35		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle moderately enlarged when killed on 14th day
	CR 6-00		2 cc. s.c. and 0.5 cc. i.t.	No subcutaneous lesion; no general symptoms; inoculated testicle slightly enlarged; complete and uneventful recovery
11 4/3/33	DR 7-09	Testicle CR 6-35	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 6th day; myxoma, probably complicated by intercurrent infection

Serial Passage of Virus myxomatosum through Cottontail Rabbits

The 17 animals furnishing the basis for the foregoing description of *Virus myxomatosum* infection in cottontail rabbits were part of an experiment in which an attempt was made to determine whether the virus would be modified by serial passage in this species.

Virus myxomatosum has been submitted to 10 serial cottontail rabbit passages over a period of 140 days. The inoculated testicle was used as a source of virus for each succeeding serial passage except the third when tissue from the subcutaneous lesion was utilized. The virus was tested at each passage by inoculation into domestic rabbits to detect whatever attenuating influence cottontail rabbit passage might exert upon it. In both the cottontail and the domestic rabbit infections only one testicle was inoculated. A record of the passage experiment is outlined in Table I.

Consideration of the data presented in Table I indicates that passage of *Virus myxomatosum* serially through cottontail rabbits did not attenuate it for domestic rabbits. Nothing to suggest conversion of *Virus myxomatosum* into the virus of infectious fibroma was revealed by the procedure. In the experiments recorded in Table I animals to be used as a source of virus were sacrificed on from the 11th to the 19th day following inoculation. From other experiments not recorded in this table, it is known that *Virus myxomatosum* persists in the infected testicles of cottontail rabbits and remains fully virulent for domestic rabbits for at least 21 days. In one instance it could not be demonstrated by animal inoculation after 32 days.

Immunological Relationship of Infectious Myxoma of Cottontail Rabbits to Infectious Fibroma.—

The sera from 6 cottontail rabbits recovered from infection with *Virus myxomatosum* have been tested for neutralizing properties against this virus. Myxoma virus from glycerolated domestic rabbit testicles was used for the experiment. It was prepared in 5 per cent suspension in 0.9 per cent NaCl solution and then centrifuged. The supernatant fluid after decantation was diluted 1:25 and 0.5 cc. of it was mixed with 1.5 cc. of each sample of cottontail rabbit serum under test. For controls, mixtures of 0.5 cc. of the same dilution of infectious suspension and 1.5 cc. quantities of normal cottontail rabbit serum were prepared. All of the mixtures were stored overnight (17 hours) prior to subcutaneous inoculation into domestic rabbits.

The results of the experiment were consistent in that all serum samples from myxoma-recovered cottontail rabbits possessed some neutralizing properties for *Virus myxomatosum*. The 2 control rabbits died in 10 and 11 days. 3 of the rabbits receiving mixtures containing convalescent serum died of characteristic infectious myxoma in 17, 19, and 34 days respectively. 1 rabbit, after an incubation period of 17 days, developed what appeared to be a mild myxoma and was found subsequently to have become immunized to *Virus myxomatosum*. The remaining 2 rabbits showed no evidence of illness and 1 of these tested later was found to be fully susceptible. 3 of the sera in the amounts used thus afforded some protection against *Virus myxomatosum* but failed to prevent fatal infection, 1 protected sufficiently well to prevent death while 2 protected completely.

Three of these sera were tested further for their ability to neutralize the virus of infectious fibroma by using 3 parts of serum to 1 part of 5 per cent testicular fibroma virus suspension. All 3 neutralized fibroma virus completely when the mixtures were tested by subcutaneous inoculation into domestic rabbits. The cottontail rabbits furnishing the 3 serum samples were inoculated subcutaneously and intratesticularly with fibroma virus, of proven infectivity by both routes for a control cottontail rabbit, and were found to be completely resistant to infection.

Immunological Relationship of Infectious Myxoma of Domestic Rabbits to Infectious Fibroma.—In an earlier paper (1) it was recorded that a single domestic rabbit upon recovery from an attack of myxoma induced by infection with an almost neutral serum-virus mixture was not only resistant to infection with the virus of infectious fibroma but also yielded a serum which neutralized both the fibroma and myxoma viruses. The exact proportions of neutralizing serum and virus necessary to produce non-fatal myxoma infections in domestic rabbits are difficult to ascertain. Most of the mixtures tried are found to contain either too much or too little serum, in which cases, respectively, the injected animal either acquires no illness and no immunity or develops myxoma and succumbs. However, out of a number of attempts, 3 other domestic rabbits have been given non-fatal attacks of myxoma by inoculation with almost neutral serum-virus mixtures.

These 3 animals were found immune to fibroma[1] and their sera capable of neutralizing both the fibroma and myxoma viruses. These experiments indicate that domestic rabbits, as well as cottontail rabbits, not only become resistant to fibroma virus following infection with *Virus myxomatosum* but also develop antibodies capable of neutralizing fibroma virus.

DISCUSSION AND SUMMARY

In the experiments presented, *Virus myxomatosum* was observed to produce only a localized fibromatous or myxomatous orchitis when injected into the testicles of cottontail rabbits. This type of disease was quite unlike the acute fatal illness which the virus caused in domestic rabbits. 10 serial passages of *Virus myxomatosum* through cottontail rabbits, covering a total elapsed time of 140 days, failed to alter its pathogenicity for domestic rabbits. Although it proved impossible to convert the myxoma virus into fibroma virus by serial passage in cottontail rabbits, it was found that these animals, recovered from myxoma, had a solid resistance to infection with the fibroma virus. Furthermore, their sera possessed neutralizing antibodies effective against the fibroma virus as well as *Virus myxomatosum.* A similar cross-immunological relationship was observed in the cases of domestic rabbits that had survived an attack of infectious myxoma.

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[1] Sir Charles Martin has kindly allowed me to refer here to his own unpublished experiments of a similar nature. He found that 5 rabbits that had survived infection induced either by contact or by conjunctival inoculation with a strain of *Virus myxomatosum* which varies in virulence from time to time were resistant to fibroma virus administered intradermally. All showed an allergic reaction 24 to 36 hours after inoculation with fibroma virus but the superficial hyperemia and swelling disappeared by the 3rd day and no fibromas developed. 5 to 8 months intervened between the recovery of these rabbits from myxomatosis and the test inoculation with fibroma virus.

INFECTIOUS FIBROMA OF RABBITS

IV. The Infection with Virus myxomatosum of Rabbits Recovered from Fibroma

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In experiments described in the preceding paper (1) it was shown that serial passage of *Virus myxomatosum* through cottontail rabbits did not modify its pathogenicity for domestic rabbits. Furthermore, cottontail as well as domestic rabbits were found to be resistant to infection with the fibroma virus after infection with *Virus myxomatosum*, and their blood sera were effective in neutralizing both the myxoma and fibroma viruses. So far as these immunological data go, they suggest the identity of the fibroma and myxoma viruses.

However, as disclosed by earlier work (2), the immunological relationship in the opposite direction was quite different. Although domestic rabbits which had recovered from the fibroma exhibited an increased resistance to infection with *Virus myxomatosum*, which is ordinarily fatal, their sera possessed no demonstrable neutralizing properties for this virus. Furthermore, the clinical and pathological pictures of the fibroma and myxoma infections in rabbits were so different that the identity of the two viruses seemed improbable. The experiments to be described in the present paper were conducted in an effort to determine the nature of the resistance to myxoma induced in domestic rabbits by infection with the fibroma virus.

Infection of Fibroma-Recovered Domestic Rabbits with Virus myxomatosum

Fourteen out of 15 fibroma-recovered domestic rabbits were reported earlier (2) to have survived infection with *Virus myxomatosum*. Since then, the number of fibroma-recovered domestic rabbits inoculated

with amounts of *Virus myxomatosum* that would ordinarily be fatal has been increased to 62 and of these 59 have survived the infection. Control animals inoculated each time have regularly succumbed. Of more than 150 normal domestic rabbits infected during this period with the same strain of *Virus myxomatosum* none survived. This uniform fatality of *Virus myxomatosum* for domestic rabbits is in accord with the experience of others investigating the disease. The minimum time for the establishment of a state of resistance to fatal infection with *Virus myxomatosum* is not known. That it is something less than a fortnight is indicated by the fact that 2 rabbits in the series were found to be resistant to fatal myxoma infection 14 days after inoculation with the fibroma virus. Rabbits tested for resistance to myxoma as late as 100 days after their primary fibroma infection proved resistant. In the cases of the 3 fibroma-recovered rabbits above mentioned that died of myxoma following inoculation with *Virus myxomatosum* 24, 35, and 56 days had elapsed between the primary inoculation with fibroma virus and the inoculation with *Virus myxomatosum*.

Repeated injections of fibroma virus failed to enhance the resistance of rabbits to myxoma or to establish antibodies neutralizing *Virus myxomatosum* in their sera. 2 rabbits that had received 2 injections and 1 that had received 4 injections of fibroma virus subcutaneously and intratesticularly were found to be no more resistant to *Virus myxomatosum* than animals receiving but a single injection. Furthermore, the sera of these 3 rabbits possessed no demonstrable neutralizing properties for *Virus myxomatosum*.

The wide time range over which infection with fibroma virus exerts its protective influence against fatal infection with *Virus myxomatosum* (from 14 to 100 days) seems to eliminate the possibility that the increased resistance is of a non-specific nature. This is further indicated by the fact that the mere injection of fibroma virus, even in large dosage, confers no protection; actual fibromatous growth is necessary. For instance, domestic rabbits injected intraperitoneally even with very large amounts of fibroma virus develop no growths and are subsequently still fully susceptible to fatal infection with *Virus myxomatosum*; on the other hand, relatively much smaller amounts of fibroma virus given subcutaneously or intratesticularly regularly result in growths and the establishment of resistance to infectious myxoma.

The Clinical and Pathological Picture of Infectious Myxoma in Fibroma-Recovered Animals.—As was pointed out previously (2), fibroma-recovered rabbits were but rarely completely resistant to *Virus myxomatosum*. Most of the animals developed myxoma in an abortive form, and the lesions, though characteristic of the disease, were limited to the formation of a localized myxomatous growth if the inoculation had been subcutaneous, or to a myxomatous orchitis if the inoculation had been intratesticular. Sometimes these local processes were accompanied by mild conjunctivitis and a purulent type of rhinitis which were transient. Myxomatous swelling of the eyelids, nose, ears, and genito-anal region developed rarely and the picture presented was that of typical acute infectious myxoma, differing from it, however, in that this condition was not fatal. In many instances in which fibroma-recovered rabbits were inoculated both subcutaneously and intratesticularly with *Virus myxomatosum*, myxomatous lesions developed only in the testicle. In respect to the general clinical picture, fibroma-recovered domestic rabbits reacted to infection with *Virus myxomatosum* in much the same manner as did normal cottontail rabbits (1). It appears that preliminary infection with fibroma virus induces in the highly susceptible domestic rabbit a resistance to *Virus myxomatosum* similar in degree to that exhibited naturally by the cottontail rabbit.

The gross and histopathological characters of the local lesions developing in the testicles or subcutaneous tissues at the site of *Virus myxomatosum* inoculation in fibroma-recovered domestic rabbits were similar to those at corresponding sites in fully susceptible domestic rabbits. The healing process seen only in resistant rabbits was characterized by a marked infiltration of the local lesions with round cells. Cytoplasmic acidophilic inclusions were present in epithelial cells of the epididymis of the inoculated testicle and in those of the epidermis overlying local growths in the subcutaneous tissue in resistant rabbits. These were identical in appearance with the inclusions seen in similar cells of fully susceptible rabbits.

Recovery of Virus myxomatosum from the Local Myxomatous Lesions Induced in Fibroma-Recovered Rabbits and Its Passage in Series through Such Animals.—Preliminary experiments showed that when fibroma-recovered domestic rabbits were inoculated subcutaneously or intratesticularly with *Virus myxomatosum* and developed only a local myxomatous lesion, myxoma virus, fully pathogenic for normal rabbits could be recovered from such lesions even as late as 16 days after inoculation. The local lesions by this time were often regressing. This rendered likely the possibility that the virus might prove serially transmissible in such animals.

In order to study the relationship of fibroma to myxoma virus, and because of the possibility of altering the pathogenic properties of

Virus myxomatosum, it seemed advisable to attempt the serial passage of this virus through fibroma-recovered rabbits. Further, the question first raised by Rivers (3) of whether *Virus myxomatosum* is a single virus or composed of more than one virus might be answered. For instance the immunological relationships between the fibroma and the myxoma viruses, outlined earlier in this paper, were in accord with the possibility that *Virus myxomatosum* might be composed of fibroma virus and some other perhaps hitherto unknown virus. If such were the case, it could easily be understood why *Virus myxomatosum* would immunize completely against the fibroma virus, one of its components, while the fibroma virus, being but one part of *Virus myxomatosum*, gives correspondingly only partial immunization.

In the serial passage of *Virus myxomatosum* through fibroma-recovered rabbits at least one normal control rabbit was inoculated at each passage to detect any change in the character of the disease induced by the virus. Male rabbits were used and, in the cases of the resistant rabbits, the preliminary inoculation with fibroma virus had been made subcutaneously and into one testicle. In inoculating such animals subsequently with *Virus myxomatosum* the other testicle and a new subcutaneous site were chosen. Fresh *Virus myxomatosum* from the subcutaneous lesion of a rabbit dead of the disease was used in starting the experiment. At each passage the infected testicle from a resistant rabbit was used in inoculating animals of the succeeding passage. Usually the testicle was removed, under ether anesthesia, on from the 10th to the 12th day following inoculation, thus allowing the animal furnishing the virus to recover and complete its record in the experiment. In the first 2 passages, the animals serving as the source of passage virus were killed. No virus for passage was taken from a resistant rabbit until the normal control animal had died. *Virus myxomatosum* was passed in this manner through fibroma-recovered rabbits for 8 serial passages at which time the experiment was discontinued. An outline of the experiment is presented in Table I.

Consideration of the data given in Table I reveals that *Virus myxomatosum* was readily transmissible in series through the testicles of fibroma-recovered rabbits. Its pathogenic properties, as judged by inoculation into normal domestic rabbits, were unchanged by such passage. None of the fibroma-recovered rabbits used in the experiment died and most showed only a localized testicular myxomatosis.

Rous, McMaster, and Hudack (4) have shown that living cells protect viruses associated with them from the neutralizing effect of immune serum. The possibility, suggested by this work, that living

cells in the inoculum administered at each serial passage served to shield the virus from neutralization in the resistant animals and thus perpetuate it from passage to passage was shown not to be a factor; myxoma virus from infected testicles stored for 2 months in 50 per cent glycerol at refrigerator temperature induced localized myxomatous orchitis when administered intratesticularly to fibroma-recovered rabbits and virus was demonstrable in such local lesions.

The Disappearance of Virus myxomatosum from the Site of Inoculation in Immune Rabbits.—An animal wholly immune to a virus not only yields a specific neutralizing blood serum and is completely refractory to reinfection but in addition, when reinoculated, is capable of rendering injected virus rapidly non-demonstrable. The failure to recover virus from the sites of inoculation in immunized animals has been reported repeatedly.

Kraus, Keller, and Clairmont (5) demonstrated that rabies virus could not be got from the brains of immunized rabbits 5 days following inoculation, and Kraus and Doerr (6) found that while fowl plague virus was still demonstrable in the brains of immunized geese 6 hours following inoculation, this was not the case 18 hours after injection. Levaditi and Nicolau (7) observed that vaccinia virus inoculated into the brain of an immunized rabbit could not be demonstrated 2 hours following injection. Andrewes (8) showed that Virus III inoculated into the testicles of immunized rabbits was not to be recovered 2 hours after injection and Nicolau and Kopciowska (9) made a similar observation regarding herpes virus introduced into the brains of immunized rabbits. Smith (10) noted that while vaccinia virus persisted in the circulation of a susceptible rabbit for as long as 8 days following intravenous infection, it disappeared from the circulation of an immune rabbit within 4 to 6 hours.

The following experiment was performed in an attempt to demonstrate a similar phenomenon in animals immune to *Virus myxomatosum*.

Three male rabbits were made resistant to infectious myxoma by a preliminary subcutaneous infection with fibroma virus. They were next submitted to a subcutaneous inoculation of *Virus myxomatosum*. A local myxomatous lesion developed in the subcutaneous tissue at the site of inoculation. Following regression of the lesion, the sera of these rabbits contained demonstrable neutralizing antibodies for *Virus myxomatosum* and the animals were deemed immune. They were then inoculated subcutaneously and into each testicle with a suspension of *Virus myxomatosum* from glycerolated infected testicles. As controls, a normal and a fibroma-recovered rabbit received the virus in a similar manner. The normal

TABLE I

Passage of Virus myxomatosum Serially through Fibroma-Recovered Rabbits

Passage No. and date	Domestic Rabbit No.	Previous treatment	Infection with *Virus myxomatosum*		Result
			Supernatant of a 5 per cent suspension testicle Rabbit No.	Dosage and route of inoculation	
1 10/31/32	4-72	None (control)	1:50 dilution 5 per cent suspension of subcutaneous myxomatous lesion Rabbit 4-81	1 cc. s.c.* and 0.2 cc. i.t.*	Died, 11th day; typical myxoma
	4-56	Subcutaneous fibroma		1 cc. s.c.	No general symptoms; slight local subcutaneous lesion; recovered
	4-65	Subcutaneous and testicular fibroma		1 cc. s.c. and 0.2 cc. i.t.	No general symptoms; inoculated testicle moderately enlarged; recovered
	4-74	Subcutaneous and testicular fibroma		1 cc. s.c. and 0.2 cc. i.t.	No general symptoms; inoculated testicle moderately enlarged when killed on 11th day
2 11/11/32	5-04	None (control)	4-74	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 8th day; typical myxoma
	4-66	None (control)		0.5 cc. s.c. and 0.1 cc. i.t.	Died, 8th day; typical myxoma
	4-71	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	No general symptoms; slight local subcutaneous lesion; inoculated testicle moderately enlarged; recovered
	4-61	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	No general symptoms; slight local subcutaneous lesion; inoculated testicle moderately enlarged when killed on 11th day

3 11/22/32	5-13	None (control)	4-61	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 9th day; typical myxoma
	4-87	None (control)		0.5 cc. s.c. and 0.1 cc. i.t.	Died, 9th day; typical myxoma
	4-77	Subcutaneous fibroma		0.5 cc. s.c.	No local lesion or illness
	4-78	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	No general symptoms; no subcutaneous lesion; inoculated testicle moderately enlarged when removed on 10th day;† recovered
4 12/ 2/32	4-44	None (control)	4-78	0.5 cc. s.c.	Died, 10th day; typical myxoma
	4-68	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	No general symptoms; no subcutaneous lesion; inoculated testicle moderately enlarged when removed on 11th day; recovered
5 12/13/32	5-28	None (control)	4-68	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 8th day; typical myxoma
	5-20	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	Completely negative
	5-21	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	No general symptoms; no subcutaneous lesion; inoculated testicle moderately enlarged when removed on 11th day; recovered
6 12/24/32	5-27	None (control)	5-21	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 11th day; typical myxoma
	5-23	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	Clinical picture characteristic of myxoma; recovered
	5-16	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	No general symptoms; no subcutaneous lesion; inoculated testicle slightly enlarged and firm when removed on 11th day; recovered

* s.c. = subcutaneously; i.t. = intratesticularly into one testicle.

† All operative procedures were conducted under full ether anesthesia.

TABLE I—*Concluded*

Passage No. and date	Domestic Rabbit No.	Previous treatment	Infection with *Virus myxomatosum*		Result
			Supernatant of a 5 per cent suspension testicle Rabbit No.	Dosage and route of inoculation	
7 1/ 4/33	5-77	None (control)	5-16	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 8th day; typical myxoma
	5-24	Subcutaneous fibroma		0.5 cc. s.c.	No general symptoms; moderate local subcutaneous myxomatous lesion
	5-17	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	Completely negative
	5-50	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	No general symptoms; no subcutaneous lesion; inoculated testicle greatly enlarged when removed on 11th day; recovered
8 1/15/33	5-71	None (control)	5-50	0.5 cc. s.c. and 0.1 cc. i.t.	Died, 12th day; typical myxoma
	5-22	Subcutaneous and testicular fibroma		0.5 cc. s.c. and 0.1 cc. i.t.	No general symptoms; scant local subcutaneous lesion; inoculated testicle slightly enlarged and firm when killed on 12th day
9 1/27/33	5-72	None	5-22	0.5 cc. s.c.	Died, 13th day; typical myxoma

control rabbit developed typical myxomatosis and died on the 9th day. The fibroma-recovered control developed localized subcutaneous and testicular myxomata, a transient conjunctivitis, and survived. The 3 myxoma-immune animals remained normal. A testicle was removed, under ether anesthesia, from each of the control rabbits 20 hours following infection, and from the myxoma-immune animals 20, 48, 72, and 96 hours following inoculation. The testicles were ground in a mortar, suspended in physiological saline, and a portion of each suspension thus prepared was inoculated subcutaneously and intratesticularly into rabbits to test for the presence of *Virus myxomatosum*. *Virus myxomatosum*, inducing typical infectious myxoma fatal in 9 and 10 days, respectively, was demonstrable in the testicles removed at the end of 20 hours from the normal and fibroma-recovered control rabbits. The testicles removed 20, 48, 72, and 96 hours following inoculation from the myxoma-immune rabbits were free from *Virus myxomatosum* demonstrable by rabbit inoculation.

The results of this experiment suggest that, as in other virus diseases, virus inoculated into the tissues of rabbits immune to infectious myxoma is promptly destroyed or rendered non-demonstrable.

The Failure of Virus myxomatosum to Invade the Blood Stream of Fibroma-Recovered Rabbits.—In infectious myxoma of rabbits the etiological virus invades the blood stream and is regularly found there throughout the later course of the disease (11 and 12). It seemed of interest to determine whether it was similarly present in the blood stream of fibroma-recovered rabbits after inoculation with *Virus myxomatosum*.

Three fibroma-recovered rabbits that developed a localized myxomatous orchitis after intratesticular inoculation with *Virus myxomatosum* were bled from the ear vein on the 2nd, 4th, 7th, and one on the 11th day after infection. Serum from each of these bleedings failed to produce infectious myxoma in test rabbits to which it was administered subcutaneously in 3 cc. amounts. Similar amounts of serum obtained from non-resistant rabbits, infected with myxoma, from the 7th day post-infection to death regularly produced infectious myxoma in test rabbits to which it was similarly administered.

These experiments indicated that *Virus myxomatosum* did not invade the blood stream in fibroma-recovered rabbits as it did in fully susceptible animals. They did not, however, shed light on the actual mechanism by which *Virus myxomatosum* is restrained to a localized and relatively benign infection in fibroma-recovered rabbits. This will be considered in more detail later.

TABLE II

The Time of Appearance of Myxoma Neutralizing Antibodies in the Blood Serum of Fibroma-Recovered Rabbits Infected with Virus myxomatosum

Serum from Rabbit No.	Drawn, days after inoculation with *Virus myxomatosum*	Effect of subcutaneous injection of mixture of 0.5 cc. 1:25 dilution of *Virus myxomatosum** + serum		
		Amount of serum in mixture	Injected Rabbit No.	Result
		cc.		
	Fibroma-convalescent (before myxoma)	3	8-99	Died, 15 days
	2 days	3	8-91	Died, 17 "
	4 "	3	8-85	Died, 17 "
8-65	7 "	3	8-81	No illness
	10 "	3	9-04	No illness
	15 "	1.5	9-41	No illness
	46 "	1.5	9-39	No illness
	Fibroma-convalescent (before myxoma)	3	9-00	Died, 13 days
	Fibroma-convalescent (before myxoma)	3	9-54	Died, 11 "
	2 days	3	8-83	Died, 17 "
8-66	4 "	3	8-89	Died, 11 "
	7 "	3	8-84	No illness
	10 "	3	9-03	No illness
	15 "	3	9-55	No illness
	46 "	1.5	9-43	No illness
	Fibroma-convalescent (before myxoma)	3	8-98	Died, 12 days
	Fibroma-convalescent (before myxoma)	5	9-52	Died, 12 "
	2 days	3	8-86	Died, 22 "
	2 "	5	9-40	Died, 13 "
	4 "	3	9-05	Died, 18 "
	4 "	5	9-46	Died, 16 "
8-70	7 "	3	8-88	Died, 17 "
	7 "	5	9-42	Died, 13 "
	10 "	3	9-02	Died, 13 "
	10 "	5	9-57	Died, 25 " (probably not of myxoma)
	15 "	5	9-55	No illness
	46 "	5	9-53-A	No illness
	Fibroma-convalescent (before myxoma)	3	11-03	Died, 18 days
10-57	7 days	3	11-00	Died, 15 "
	17 "	3	11-24	No illness

* *Virus myxomatosum* = supernatant of a 5 per cent suspension of glycerolated testicle and subcutaneous lesion from rabbit dead of infectious myxoma.

TABLE II—*Concluded*

Serum from Rabbit No.	Drawn, days after inoculation with *Virus myxomatosum*	Effect of subcutaneous injection of mixture of 0.5 cc. 1:25 dilution of *Virus myxomatosum* + serum		
		Amount of serum in mixture	Injected Rabbit No.	Result
		cc.		
	Fibroma-convalescent (before myxoma)	3	11-05	Died, 15 days
10-58	7 days	3	10-99	No illness
	17 "	3	11-20	No illness
	Fibroma-convalescent (before myxoma)	3	11-01	Died, 16 days
10-69	7 days	3	10-98	No illness
	17 "	3	11-21	No illness
	Fibroma-convalescent (before myxoma)	3	11-22	Died, 17 days
10-70	7 days	3	10-97	Died, 13 "
	17 "	3	11-23	No illness
8-55	Normal	3	8-96	Died, 14 days
14	Normal	5	9-53-B	Died, 10 "
14	Normal	3	10-35	Died, 11 "
10-74	Normal	3	11-07	Died, 16 "
10-74	Normal	3	11-19	Died, 20 "

The Time of Appearance of Myxoma-Neutralizing Antibodies in the Blood Serum of Fibroma-Recovered Rabbits Infected with Virus myxomatosum.—In the preceding section it was noted that *Virus myxomatosum* could not be detected in the serum of myxoma-infected fibroma-recovered rabbits. It seemed possible that the failure of the virus to generalize in these animals might be due to their prompt generation of virus-neutralizing antibodies. Such an occurrence would aid in explaining the benign and localized nature of their myxoma infections. Therefore, the time of appearance of myxoma-neutralizing antibodies in the blood serum of infected fibroma-recovered rabbits was investigated.

A series of rabbits was bled following recovery from infection with the fibroma virus. They were then inoculated either subcutaneously or intratesticularly, or by both routes, with *Virus myxomatosum* in amounts large enough to kill all control animals. Serum was obtained from 3 of the rabbits on the 2nd, 4th, 7th, 10th, 15th, and 46th days and from the remaining 4 on the 7th and 17th days following infection with *Virus myxomatosum*. This serum, together with that obtained prior to their infection, was then tested for its ability to neutralize *Virus myxomatosum*.

The neutralization tests were conducted in the usual fashion. The serum-virus mixtures were set up to contain 0.5 cc. of a 1:25 dilution of the supernatant of a 5 per cent suspension of glycerolated subcutaneous and testicular myxoma lesion (the equivalent of 1 mg. of infectious tissue) mixed with the amount of serum being tested, usually 3 cc. The mixtures were stored overnight (17 hours) in the refrigerator prior to injection subcutaneously into the test rabbits. Rabbits receiving mixtures which were neutral developed no evidence of infectious myxoma and survived. Those receiving mixtures in which the serum did not neutralize the virus came down with the typical disease and died in from 11 to 20 days following inoculation. These rather long survival periods are believed to be the result of the relatively small amounts of virus employed in the mixtures. The amount of virus used was, however, sufficient to kill all control rabbits. The results of the neutralization experiments are outlined in Table II.

The data recorded in Table II reveal that of the serum samples obtained prior to the 7th day following infection with *Virus myxomatosum* none neutralized the virus. Of 7 of the samples obtained on the 7th day following infection, however, 4 neutralized the virus completely. Of the remaining 3 rabbits, the serum of 1 failed to neutralize virus on the 10th day but did on the 15th day, while the other 2 both neutralized on the 17th day post-infection. An attempt to determine a possible relationship between the time of appearance of neutralizing antibodies and the severity of the myxoma infection leads to the impression that the promptness of antibody reaction was determined by the severity of the infection, rather than that the severity of the infection was determined by the promptness with which antibodies were produced. 3 of the 4 rabbits whose serum contained neutralizing antibodies as early as the 7th day post-infection developed either a coryza or a conjunctivitis in addition to myxomatous swellings at sites of inoculation, while 2 of the 3 rabbits in which the appearance of demonstrable antibodies was delayed until later than the 7th day post-infection exhibited no evidence of generalizing infection. The exceptional animal in each group is sufficient to indicate that any

attempt to correlate the speed of production of antibodies with the extent and severity of the disease in so small a group of experimental animals is hazardous. The fact remains, nevertheless, that fibroma-recovered rabbits produce antibodies capable of neutralizing *Virus myxomatosum* during an attack of the modified infectious myxoma that they develop. The sera of fully susceptible rabbits infected with myxoma virus at no time contains neutralizing antibodies and, as was pointed out in the preceding section, is rich in virus from the 7th day post-infection to death. The time of appearance of virus neutralizing antibodies in the sera of resistant rabbits thus approximately coincides with that at which the virus ordinarily generalizes in susceptible animals. It seems possible that this fortunate coincidence of events may be at least partially accountable for the apparent resistance of fibroma-recovered rabbits to *Virus myxomatosum*.

DISCUSSION

The transmissibility of *Virus myxomatosum* in series through fibroma-recovered rabbits without alteration of its disease-producing properties, in contrast with its failure even to survive in the tissues of myxoma-immune rabbits, is of importance as far as reaching a decision concerning the identity of the fibroma virus with *Virus myxomatosum*. The generally accepted criterion for considering two viruses identical is an immunological one. Animals recovered from infection with each virus should not only resist infection with the other virus but their sera should neutralize it. They should, furthermore, be capable of inactivating or destroying the other virus when it is introduced into an ordinarily susceptible tissue. So far as the immunological relationship between the fibroma virus and *Virus myxomatosum* is concerned these criteria of complete cross-immunity are not fulfilled. Even though infection of a rabbit with fibroma virus is known to establish in that animal a state of enhanced resistance to *Virus myxomatosum*, the fibroma-recovered rabbit is usually not completely refractory to myxoma infection as evidenced by the development of a local myxomatous lesion at the site of inoculation. Furthermore injection of *Virus myxomatosum* into a fibroma-recovered rabbit does not result in the destruction of the injected virus. On the contrary, the virus actually multiplies and can be passed indefinitely in series through

such resistant animals. Finally, serum from a fibroma-recovered rabbit, though neutralizing the fibroma virus, is without effect on *Virus myxomatosum*. It is plain that the two viruses are not identical.

The results of cross-protection and cross-neutralization experiments can be best explained on the basis of a partial duplication of the antigenic components comprising the two viruses. It was shown in the preceding paper (1) that rabbits recovered from *Virus myxomatosum* infection were immune to the fibroma virus and their sera capable of neutralizing that virus. This indicated that *Virus myxomatosum* contained antigenic components essential to the production of a complete fibroma virus immunity. On this basis, the incomplete protection of rabbits in the reverse direction might be interpreted as indicating that the fibroma virus is antigenically only a partial replica of *Virus myxomatosum*. The antigenic components comprising fibroma virus and common also to the myxoma virus are sufficient to establish in fibroma-infected rabbits a state of resistance to myxoma, but, because they represent only partially the antigenic composition of *Virus myxomatosum*, this resistance is not the complete immunity conferred reciprocally by two identical viruses.

The experimental data presented are considered to support the view, evident also from the clinical and pathological data, that, while perhaps antigenically and genetically closely related, *Virus myxomatosum* and fibroma virus are different infectious agents.

SUMMARY

The serial passage of *Virus myxomatosum* through fibroma-recovered domestic rabbits did not alter its pathogenic properties. Fully virulent *Virus myxomatosum* persisted in the inoculated testicle of fibroma-recovered rabbits for at least 16 days following inoculation. Virus injected into the testicles of myxoma-immune domestic rabbits, on the other hand, was promptly rendered non-demonstrable. The failure of fibroma-recovered domestic rabbits to destroy injected *Virus myxomatosum* and the absence from their sera of neutralizing antibodies effective against *Virus myxomatosum* are considered to be evidence against the identity of the fibroma and myxoma viruses. The rapidity with which fibroma-recovered rabbits develop neutralizing

antibodies following infection with *Virus myxomatosum* is considered to be a possible factor in their acquired resistance.

It is believed on the basis of all the evidence that infectious fibroma of rabbits is a definite disease entity and not merely a mild and non-fatal form of infectious myxoma.

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INHIBITION OF THE SHWARTZMAN PHENOMENON

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In 1928 Shwartzman described a new phenomenon of local skin reactivity to culture filtrates of various microorganisms. The reactivity was induced by the injection of a filtrate into the skin of a rabbit. If an intravenous injection of a potent bacterial filtrate was given to the same rabbit from 20 to 24 hours later, there appeared an extremely severe hemorrhagic necrosis at the site of the previous injection. The factors determining the local skin reactivity were termed skin-preparatory factors and those responsible for the local injury following intravenous injection were called reacting factors. In recent years, an extensive series of studies on the various aspects of this phenomenon has been reported by Shwartzman and other workers.

Gross (1) described briefly an observation concerning inhibition of the Shwartzman phenomenon. The inhibition was obtained when an intravenous injection of a bacterial filtrate was given to a rabbit shortly after or simultaneously with the skin-preparatory injection. No reactions followed the provocative intravenous injection of the same filtrate 24 hours later. The same phenomenon of inhibition was observed independently by the author of this paper in his experiments with *B. coli* culture filtrates (2).

The present paper embodies a series of experiments concerning the inhibition of the Shwartzman phenomenon by means of active bacterial filtrates.

Material and Methods

Typhoid and meningococcus "agar washings" filtrates used were prepared and titrated according to the methods described by Shwartzman.

B. typhosus "agar washings" filtrates (3) were prepared as follows: Kolle flasks

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containing plain veal infusion agar of pH 7.4 were seeded each with 3–4 cc. of 20 hour old plain broth culture of *B. typhosus* (strain T_I) diluted 1:4 with 0.9 per cent NaCl solution. The dilution was made immediately before use. After 20 to 22 hours of incubation the growth of each flask was washed off with 2–4 cc. of 0.9 per cent NaCl solution containing 0.4 per cent phenol. The washings were then pooled, centrifuged within the following 1 to 2 hours and the clear supernatant fluid filtered through Berkefeld V candles shortly after centrifuging.

The method of preparation of *meningococcus "agar washings" filtrates* (4) differed from the above in the following: The inoculum was prepared by inoculation of meningococcus into 1 per cent rabbit blood broth of pH 7.2 – 7.4. The supernatant broth culture, after 20 to 22 hours of incubation, free of red blood cells was used for inoculation of the Kolle flasks. The Kolle flasks contained 0.7 per cent glucose veal infusion agar.

The quantitative measurement of the reacting factors (5) was carried out as follows: The rabbits used for titrations were each injected intradermally with 0.25 cc. of the undiluted filtrate or filtrate diluted 1:2 and divided into groups of three. 24 hours later a single intravenous injection of the filtrate diluted in 0.85 per cent NaCl solution was given to each rabbit. The dose was 1 cc. per kilo of body weight. Each group of rabbits received intravenously a different dilution of the filtrate. The local reactions were read 4 to 5 hours after the intravenous injections. The titrations were carried until the lowest dilution was found which gave no reaction in the four rabbits tested, as well as the highest dilution which gave reactions in one or more rabbits of the group. The minimal dose of reacting factors was then considered as lying between these two figures. If a given filtrate was employed for any length of time, repeated control titrations were done. In these control tests, the dilutions employed were both the highest dilution capable of eliciting reactions and the lowest dilution giving no reactions.

EXPERIMENTAL

1. Titration of Skin-Preparatory Factors against 25 Reacting Units.—Preliminary experiments suggested that it was necessary to employ a minimal skin-preparatory dose for the demonstration of inhibition of the Shwartzman phenomenon. Meningococcus Group III "agar washings" filtrate (T.1968) containing 4,000 reacting units was used. The skin of rabbits was prepared with various dilutions of the filtrate. For the provocative injection 25 reacting units were used in all the groups. The results are recorded in Series 1 of Table I.

As is seen from experiments of Series 1 of Table I, severe reactions were obtained in skin sites of 80 per cent of rabbits prepared with 0.25 cc. of filtrate T. 1968 diluted 1:25 and 24 hours later injected intravenously with 25 reacting units (*i.e.*, dilution 1:100 per kilo of body weight).

2. Inhibition Tests.—Different dilutions of the meningococcus filtrate (T.1968) were prepared. 1 cc. per kilo of body weight of each dilution was injected intravenously into rabbits. Immediately afterwards a preparatory injection of 0.25 cc. of the filtrate diluted 1:25 was made into the skin of the abdominal wall. 20

TABLE I

Series No.	First intravenous injection Dilution	Intradermal injection Dilution	Second intravenous injection Dilution	Total No. of rabbits	No. of deaths	Reactions				
						4+	3+	2+	1+	Negative
1	—	Mg. 44B.* T.1968	Mg. 44B. T.1968							
		1:25	1:160	20	1	16	0	0	0	3
		1:50	"	5	0	0	0	1	1	3
		1:75	"	3	0	0	0	0	0	3
2	—	Mg. 44B. T.1968	Mg. 44B. T.1968							
		1:25	1:400	5	0	1	0	1	0	3
		"	1:1,000	5	0	0	0	0	0	5
		"	1:4,000	5	1	0	0	0	0	4
3	Mg. 44B. T.1968	Mg. 44B. T.1968	Mg. 44B. T.1968							
	1:160	1:25	1:160	18	6	1	0	1	0	10
	1:400	"	"	10	0	1	0	0	0	9
	1:1,000	"	"	5	1	1	1	0	0	2
	1:4,000	"	"	5	0	2	1	0	0	2
4	Mg. 44B. T.1968	Mg. 44B. T.1968	Mg. 44B. T.1968							
	1:160	1:10	1:160	10	3	4	0	0	0	3
	"	1:2	"	10	4	3	0	0	0	3
5	Mg. 44B. T.1968	Mg. 44B. T.1968	Mg. 44B. T.1968							
	1:160	1:25	1:80	6	2	2	0	0	0	2
	"	"	1:40	4	2	1	0	0	0	1

In this and the following tables, all intravenous injections were given in a dose of 1 cc. per kilo of body weight; all intradermal injections were given in a dose of 0.25 cc.

The intensity of hemorrhagic and necrotic lesions noted as 1+, 2+, 3+ and 4+.

* Abbreviation Mg. 44B. designates "agar washings" filtrates of meningococcus, Group III cultures.

to 24 hours after the intradermal injection, 1 cc. per kilo of body weight of the filtrate diluted 1:160 (*i.e.*, 25 reacting units) was injected intravenously. The readings of the skin reactions were made 4 to 5 hours after the last injection. The results are recorded in Series 3 of Table I.

It became obvious from these experiments that the Shwartzman phenomenon to meningococcus filtrate could be almost completely inhibited by an intravenous injection of the filtrate simultaneously with the skin-preparatory injection of the same filtrate in a dose of 0.25 cc. of dilution 1:25. The effective inhibitory doses were dilutions ranging from 1:160 to 1:400. The inhibitory effect of a dilution 1:1,000 was doubtful and higher dilutions remained without effect.

Similar experiments were carried out with increasing concentrations of the skin-preparatory dose and constant doses of the inhibitory and provocative injections. The results are recorded in Series 4 of Table I.

As is seen from these experiments, an intravenous injection of filtrate T. 1968 given simultaneously with the skin-preparatory injection of the same filtrate in dilutions lower than 1:25 failed to inhibit the Shwartzman phenomenon.

In experiments of Series 5 of Table I, constant amounts were used for the inhibitory intravenous injections (*i.e.*, dilution 1:160) and preparatory intradermal injections (*i.e.*, dilution 1:25). Rabbits thus treated were tested with various provocative doses. If the results of Series 5 are compared with those of Series 3, it is obvious that no inhibition of the Shwartzman phenomenon took place if dilutions lower than 1:160 were used for the provocative injections.

From the results thus far recorded, it becomes clear that an intravenous injection accompanying the intradermal preparatory injection may inhibit the Shwartzman phenomenon within the limits of certain quantitative relationships.

The experiments recorded below were planned to determine the optimum time relationships between the inhibitory, preparatory and provocative injections.

In the experiments of Series 1 of Table II, the intervals of time between the inhibitory intravenous injections and the preparatory intradermal injections were varied. The latter injections were given 1, 3 and 4 hours after the inhibitory intravenous injections. The provocative injections were given 20 to 24 hours after the intradermal preparatory injections.

As is seen from Series 1 of Table II, an intravenous injection of meningococcus filtrate T. 1968 diluted 1:160, given 1 hour prior to the preparatory intradermal injection, was capable of inhibiting the Shwartzman phenomenon almost as effectively as an inhibitory injec-

tion given simultaneously with the preparatory injection. An inhibitory intravenous injection given 4 hours before the preparatory intradermal injection remained without effect. A 3 hour interval between the inhibitory and preparatory injections gave only irregular results. It can be stated, then, that inhibitory effect of an intravenous injection of a bacterial filtrate is of short duration.

In experiments of Series 2 of Table II, the intradermal preparatory injections were given prior to the inhibitory intravenous injections. The number of animals

TABLE II

Series No.	First intravenous injection Dilution	Interval of time	Intradermal injection Dilution	Second intravenous injection Dilution	Total No. of rabbits	No. of deaths	Reactions 4+	3+	2+	1+	Negative
		hrs.									
1	Mg. 44B. T.1968 1:160 " "	1	Mg. 44B. T.1968 1:25 " "	Mg. 44B. T.1968 1:160 " "	5	0	1	0	0	0	4
		3			5	1	1	0	1	0	2
		4			5	2	3	0	0	0	0
	Intradermally 0.25 cc. one area		First i.v. injection 1 cc. per kilo	Second i.v. injection 1 cc. per kilo							
2	Mg. 44B. T.1968 1:25 " "	1	Mg. 44B. T.1968 1:160 " "	Mg. 44B. T.1968 1:160 " "	5	1	1	0	0	0	3
		2			5	2	1*	0	0	0	2
		4			5	2	1*	0	0	0	2

* Already positive before the second intravenous injection.

employed in these experiments was rather small. The facts suggest, however, that an intravenous injection given 1 hour after the preparatory intradermal injection may inhibit the Shwartzman phenomenon. Inhibitory intravenous injections given at longer periods of time, *i.e.*, 2 and 4 hours after the intradermal injection, apparently produced no effect. In some rabbits the state of reactivity was already induced several hours after the preparatory injections. For this reason, the intravenous injections intended to inhibit the Shwartzman phenomenon served as provocative injections and elicited reactions in prepared sites. The fact introduced difficulties in the interpretation of the results of experiments with the 4 hour interval of time between skin-preparatory and intravenous injection.

TABLE III

Series No.	First intravenous injection Dilution	Intradermal injection Dilution	Second intravenous injection Dilution	Total No. of rabbits	No. of deaths	Reactions 4+	3+	2+	1+	Negative
1	B.TyT$_L$* T.1976 1:16	Mg. 44B. T.1968 1:25	Mg. 44B. T.1968 1:160	10	5	0	2	1	0	2
	B.TyT$_L$ T.1986 1:28	"	"							
	1:56	"	"	5	2	1	0	0	0	2
	1:100	"	"	11	4	3	0	0	0	4
	1:400	"	"	15	0	2	0	0	0	13
				5	1	2	0	1	0	1
2	—	Mg. 44B. T.1968 1:25	B.TyT$_L$ T.1976 1:16	5	0	4	0	0	0	1
	—	"	B.TyT$_L$ T.1986 1:28	5	0	2	0	1	0	2
	—	"	1:56	5	0	3	0	0	0	2
	—	"	1:100	5	1	4	0	0	0	0
			1:800	5	0	1	1	0	0	3
3	*B. coli* filtrate T.1964, 1:3	Mg. 44B. T.1968 1:25	Mg. 44B. T.1968 1:160	14	7	2	0	0	0	5
	1:15	"	"	10	3	2	0	0	0	5
	1:30	"	"	5	0	1	0	0	0	4
	1:100	"	"	10	3	4	0	0	0	3
	1:400	"	"	5	0	4	0	0	0	1
4	—	Mg. 44B. T.1968 1:25	*B. coli* filtrate T.1964, 1:3	5	0	3	2	0	0	0
	—	"	1:15	5	0	2	0	1	0	2
	—	"	1:30	5	0	0	0	0	0	5
5	*Streptococcus hemolyticus* filtrate T.1983, undiluted	Mg. 44B. T.1968 1:25	Mg. 44B. T.1968 1:160	5	0	2	1	1	0	1
	Streptococcus hemolyticus filtrate T.1989, undiluted	"	"	3	0	1	1	0	0	1
6	Plain broth 1:2 saline	Mg. 44B. T.1968 1:25	Mg. 44B. T.1968 1:160	10	3	4	1	0	0	2
	Horse serum (H.693) undiluted	"	"	5	1	3	0	0	0	1

* Abbreviation B.TyT$_L$ designates "agar washings" filtrates of *B. typhosus*, strain T$_L$ cultures.

As is seen from the results of the above group of experiments, the inhibitory effect of an additional injection may not last longer than 2 hours. It seems to be definitely effective, however, if it is given before the skin preparation takes place.

It is well known that the Shwartzman phenomenon can be elicited by combined injections of heterologous bacterial filtrates. It seemed of interest to determine whether there existed any specificity in the inhibition described.

In the experiments recorded in Series 1, 3, 5 and 6 of Table III, the Shwartzman phenomenon was elicited by means of meningococcus "agar washings" filtrates T.1968 (*i.e.*, intradermally, dilution 1:25 and intravenously, dilution 1:160). The inhibition was attempted by means of additional intravenous injections of *B. typhosus* T_L "agar washings" filtrate (T.1976 and T.1986, Series 1, Table III); *B. coli* "agar washings" filtrates (Series 3, Table III); *Streptococcus hemolyticus* filtrate T.1983 (Series 5, Table III); plain broth, normal horse serum and physiologic saline solution (Series 6, Table III).

As is seen from the experiments of Table III, no specificity of the inhibition reaction described could be observed. *B. typhosus* culture filtrate in dilution 1:100 was able to inhibit almost completely the Shwartzman phenomenon to meningococcus filtrate. Similarly, *B. coli* filtrate was effective in dilutions 1:30 and 1:100. As is also seen from Table III, additional intravenous injections of *Streptococcus hemolyticus* filtrate and of non-bacterial substances (*i.e.*, plain broth, normal horse serum and physiologic saline solution) failed to inhibit the Shwartzman phenomenon. In preliminary experiments not described in this paper, these agents were also shown to be lacking in reacting potency.

The facts suggest that the inhibitory effect of an additional intravenous injection can be obtained only with substances potent in the elicitation of the Shwartzman phenomenon itself.

COMMENT

It appears from the above experiments that the Shwartzman phenomenon can be inhibited if an additional intravenous injection of a potent bacterial filtrate is given within a certain period of time prior to or following the skin-preparatory injection. The inhibitory effect of the additional intravenous injection takes place within the limits of

certain quantitative relationships. Thus, if the skin is prepared with a large amount of filtrate, the inhibition is absent or incomplete. Similarly, if a large amount of filtrate is used for the provocative injection, there occurs no inhibition. It is also obvious that the inhibition described is of a transitory nature. The additional intravenous injection given several hours before or after the skin-preparation has no inhibitory effect. The inhibition can be obtained only with filtrates capable of eliciting the Shwartzman phenomenon. Bacterial filtrates of low reacting potency (*Streptococcus hemolyticus* filtrate employed in these experiments) as well as non-bacterial substances (*i.e.*, 0.85 per cent NaCl solution, plain broth and normal horse serum) produce no inhibition.

The mechanism of the inhibition remains unknown. Several explanations suggest themselves. Duran-Reynals (6) reported on the presence of spreading factors in bacterial filtrates capable of enhancing tissue permeability. It is possible that an intravenous injection of a bacterial filtrate accompanying the skin preparation may enhance the rate of diffusion of the material injected locally through increase in the capillary or lymphatic permeability. The preparatory factors thus diluted might not bring about a state of reactivity. Gross and histological examination of the tissue where reactions had been inhibited did not disclose, however, any spreading phenomenon. Further studies are under way.

Recently, experiments on generalized Shwartzman phenomenon have been described by Gratia and Linz (7), Apitz (8) and Gerber (9). The phenomenon was elicited by means of two intravenous injections, 24 hours apart. While the work reported in this paper was in progress, Apitz suggested the following explanation for inhibition of the local Shwartzman phenomenon:

The inhibitory intravenous injection may elicit a state of reactivity in the internal organs. Following the second intravenous injection reactions would occur in organs thus prepared. If a small dose is used for the provocative injection, the amount injected may be consumed in the production of lesions in the internal organs and thus an insufficient amount would remain for the elicitation of the reaction in the skin.

To determine whether this assumption could be used for the explana-

tion of the inhibition here described, the internal organs of a large series of rabbits were examined in the gross and microscopically. Rabbits showing complete inhibition of the local Shwartzman phenomenon showed only rarely lesions in the internal organs, possibly because the doses employed were too small for the elicitation of the generalized Shwartzman phenomenon.

The inhibition of the Shwartzman phenomenon cannot be interpreted as an anaphylactic desensitization for the following reasons.

1. Inhibition takes place if the additional intravenous injection is given simultaneously or shortly before and after the preparatory injection. Obviously an anaphylactic desensitization cannot be expected to occur before sensitization is induced.
2. There is no specificity of inhibition.

A great deal of experimental evidence in the literature supports the possibility that processes exemplified by the Shwartzman phenomenon take place in induced and spontaneous bacterial and virus infections. The factors responsible for the Shwartzman phenomenon and deriving from infected foci may induce a state of reactivity in tissues and organs removed from the sites of initial infection. When the state of reactivity establishes itself, discharge of the same factors into the blood stream would then elicit severe hemorrhagic lesions in these reactive sites. The mechanism might then be responsible for pathological lesions scattered through the body, and the inhibitory reaction described in this paper might prevent their occurrence.

SUMMARY

The Shwartzman phenomenon can be inhibited by an intravenous injection of a potent bacterial filtrate within a few hours before or after the preparatory intradermal injection.

The inhibitory effect is produced non-specifically by filtrates potent in the elicitation of the Shwartzman phenomenon, and it is of a transitory nature.

The relation of the observation described to anaphylactic desensitization and to its clinical significance is discussed in this paper.

The author wishes to express his gratitude to Dr. Gregory Shwartzman for the opportunity to carry out most of these experiments in his laboratory.

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IMMUNOLOGICAL AND CHEMICAL INVESTIGATIONS OF VACCINE VIRUS

III. Response of Rabbits to Inactive Elementary Bodies of Vaccinia and to Virus-Free Extracts of Vaccine Virus

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In the majority of instances human beings or lower animals that have recovered from a virus infection are free from further molestation by the same agent. This immunity may endure throughout an individual's life. Vaccinations with attenuated viruses that are still active have also been shown to induce protection. In certain cases, *e.g.*, vaccination against smallpox, such measures regularly produce a mild infection. Because of objections that have been brought against the production of disease, even though mild, by vaccination, many attempts have been made in the past to obtain serviceable amounts of protection with vaccines that cause no evidences of disease. The results of this type of work have led to differences of opinion regarding tests of complete inactivation of viruses and the antigenicity of such inactivated materials. It is not surprising that much of the work has been conducted with the virus of vaccinia. The reports which are pertinent to the present investigation will be scrutinized in some detail because of the very great practical possibilities inherent in successful methods of immunizing against the viruses.

A number of investigators (1–17) have relied on the action of heat to inactivate vaccine virus, while others (26–29) employed chemical agents such as methylene blue, bile, and chloroform. Inasmuch as our experiments deal with the antigenicity of formalin-treated vaccine virus and virus-free extracts of vaccine virus, we shall call attention only to the reports dealing with results of work in this field.

Hunt and Falk (1) allowed vaccine virus to remain in contact with 0.1 per cent formalin for 12 hours and cutaneous inoculation of the material in rabbits did not produce lesions of vaccinia. Subcutaneous injections of large amounts of the treated virus were made in rabbits and the animals treated in this manner were

found to be immune. Gordon (2) found that the immunity following injections of virus suspension rendered noninfectious by the addition of 1 per cent phenol was less than that produced by injections of heat-killed vaccine. Both kinds of treated virus were shown to be inactive by dermal inoculations in rabbits. Kraus (18) added 0.5 per cent phenol and 0.3 per cent formalin to different portions of vaccine virus and then tested them by means of application on the scarified skin of a rabbit to determine whether inactivation was complete. He found that monkeys and rabbits receiving the treated virus were partially and completely immune, respectively. Bussel and Mayzner (19) added 0.1 per cent formaldehyde to vaccine virus, and held the mixture at 37°C. for 6 weeks. Tests for the presence of active virus were not mentioned. They report that children receiving the material subcutaneously were partially immune, while those who were injected intracutaneously had unusually severe reactions to subsequent vaccinations with active virus. In rabbits, Bland (20) obtained irregular results with vaccine virus inactivated by the addition of 0.1 per cent formaldehyde or 1 per cent phenol. In guinea pigs, however, he was able regularly to induce immunity with similar materials. He tested for the presence of active virus by subcutaneous inoculations of some of the material in a guinea pig followed by 2 serial passages. Biglieri (21) was unable to demonstrate an immunity in rabbits that had received formolized (0.3 per cent) virus. However, preparations inactivated by phenol (1 per cent) or ether induced immunity. Presumably, by cutaneous inoculation in rabbits, the preparations were shown to contain no active virus. In the case of phenol and ether, a long period of time was required to accomplish inactivation. Iwanoff (22) used calf lymph to which had been added various amounts of formaldehyde. Complete inactivation of the material was demonstrated by corneal inoculations in guinea pigs. Varying degrees of immunity were obtained in rabbits and guinea pigs by injections of the treated virus. Gastinel, Reilly, and Mortier (17) added 0.2 per cent of formol to vaccine virus. Failure of an eruption to appear after inoculation of such material on the skin of a rabbit was taken to indicate inactivation of the virus. Rabbits inoculated 7 times at intervals of 5 days with the treated virus were still entirely susceptible to cutaneous inoculations with active virus, although serum taken from the animals after the injections was capable of neutralizing large amounts of virus. Hilgers (23) exposed vaccine virus to the action of 0.3 per cent formalin at 37°C. for 5 days. Such material failed to infect the cornea of a rabbit and did not produce a testicular reaction. Rabbits injected subcutaneously with the treated virus either once or several times were later tested by dermal inoculation of potent virus. In one experiment most of the animals were found resistant to inoculation of active virus; in most of the animals of other experiments the course of the vaccinal infection was only slightly modified. Hilgers considers that the virus treated in this way retains some of its antigenicity, but that its immunizing action is too irregular to be relied upon for general use.

Kramer (24) passed testicular vaccine virus through specially prepared basic filters which he had previously shown to hold back vaccine virus. He does not

mention tests for the presence of active virus in the filtrates. Rabbits were inoculated with the filtrates, and if more than 7 daily injections were made, resistance to potent virus resulted. Recently, Salaman (25) used Seitz filtrates of dermal vaccine virus and found that a partial immunity developed in rabbits as a result of several injections. He states that only the filtrates proved to be free from active virus by cutaneous inoculations of guinea pigs were used for immunization.

It is obvious that the reports of results obtained with "inactive" vaccine virus are conflicting, and in the light of our present knowledge of viruses it is doubtful whether such reports are of value to one attempting to solve the problem of the antigenicity of these agents in an inactive state. In almost all cases, emulsions of infected tissues have served as the source of the vaccine virus. In such a menstruum the virucidal action of heat and chemical agents is irregular, because protein and cellular detritus present in a tissue emulsion serve as protective substances for the virus. Furthermore, the degree of heat employed, or concentration of chemical agents added has usually been only slightly, if at all greater than the minimum requirement for inactivation of the virus as determined in other experiments. Moreover, in the instances in which tests of the treated material for the presence of active virus have been described, such tests have consisted of the inoculation of only a small portion of the emulsions in guinea pigs or rabbits, whereas for purposes of immunization large amounts of the materials were used. Such a procedure, therefore, can hardly be accepted as a conclusive demonstration of the absence of small amounts of active virus from the emulsions used for immunization. Finally, according to published reports, few or no precautions were taken to prevent the accidental transmission of vaccinia to the experimental animals from others frankly infected with the malady and housed in proximity to them. That such transmission occurs readily has been shown by previous workers (30), and may take place even when careful attempts are made to prevent it, as will appear later in the present report.

Recent work (31, 32) on the purification of the elementary bodies of vaccinia has made possible an accurate study, under properly controlled conditions, of the response to these bodies in an inactive state. It is with this problem that the present paper deals. That vaccine virus is intimately associated with these bodies is shown by the fact that suspensions of them are highly infectious (32).

Methods and Materials

Virus.—The manner in which suspensions of relatively pure elementary bodies of vaccinia were obtained for the experiments has been described in detail in a previous communication (32). At this time it is sufficient to state that such preparations contain practically nothing but elementary bodies in a state of dispersion compatible with an even and regular inactivation by heat or formalin.

Inactivation of Virus.—The elementary bodies were inactivated either by formaldehyde or heat. Experience led us to use formalin and to test for the presence of active virus in treated materials in the following manner. To large quantities of elementary body suspensions sufficient formalin was added to make the final concentration of formaldehyde 0.3 per cent. The mixture of elementary bodies and formalin was allowed to remain in a refrigerator for 10 days. Then 10 cc. were removed, and the elementary bodies after sedimentation in an angle centrifuge were resuspended in 1 cc. of Locke's solution and injected into the testicle of a rabbit. 4 days later the testicle was removed, and an emulsion was prepared, 1 cc. of which was injected into the testicle of another rabbit. 4 days later another testicular passage was made. None of the rabbits evidenced signs of a vaccinal infection and when tested for immunity to vaccinia some weeks later were found to be fully susceptible. Another 10 cc. portion of the treated elementary bodies were handled in a manner similar to that described above with the exception that the testicular passages were made at 6 day intervals instead of 4. From these rabbits also no evidence of the presence of active vaccine virus in the treated material was found. Thus 20 cc. of the formalin-treated suspension of elementary bodies were tested for the presence of active virus. In none of the experiments on immunity to be described were more than 18 cc. of this material injected into a rabbit. Consequently a greater amount was tested each time than was used for immunization of a rabbit. In some experiments the elementary bodies were concentrated and then enough formaldehyde was added to make a final concentration of 10 per cent. After standing in the ice box 10 days the material underwent a tenfold dilution before being used. Other preparations of elementary bodies were inactivated by heat. To accomplish this, the desired amount of material was sealed in large ampoules which were then immersed in boiling water for 2 hours.

Soluble Antigens.—The method by which virus-free extracts of tissues containing soluble antigens were prepared has also been described in a previous paper (32). The extracts of vaccinal infected tissue, either dermal or testicular, were freed from virus by filtration through collodion membranes[1] which had an average pore diameter of 103.0 $\mu\mu$. These filtrates were then tested for presence of active virus in the manner described above and found to contain none. They did contain the soluble antigens however, as demonstrated by means of the precipitin reaction.

[1] The membranes were prepared by Dr. J. H. Bauer of the Yellow Fever Laboratory of the International Health Division, Rockefeller Foundation.

Animals.—Healthy, full grown rabbits were used in all of the experiments.

Housing of Animals.—In order to avoid the accidental transmission of vaccinia to rabbits used in the experiments, animals were kept during the period of immunization in a separate room not previously used for work on vaccinia, and rigid precautions were taken to prevent the introduction of active virus into this room. After immunization and just prior to the inoculation with active virus for the purpose of testing their resistance, the animals were transferred to a room used for work on vaccinia.

Injections.—As a rule a 6 weeks course of injections was given, the rabbits being inoculated intraperitoneally on 2 successive days of each week with increasing amounts of material, *viz.*, 0.25, 0.50, 1.0, 2.0, 2.5, and 3.0 cc.

Controls.—To control the technic of isolation, and to test the effect of heterologous antigens, certain rabbits were given no injections, while others were inoculated with typhoid vaccine or with meat infusion broth.

Tests for the Presence of Antibodies in the Sera.—Samples of serum were collected from each rabbit at different times in order to test for the development of agglutinins, precipitins, and neutralizing antibodies. The method of conducting the agglutinin and precipitin reactions has already been described (31, 32). Neutralization tests were carried out in the following manner. A virus suspension was prepared by grinding with alundum the testicles of a rabbit inoculated 4 days previously with vaccine virus. 20 cc. of Locke's solution were added; the resulting emulsion was centrifuged at 1500 R.P.M. for 10 minutes; the supernatant liquid, designated as "undiluted virus suspension," was removed with a pipette and saved. Serial tenfold dilutions of the virus emulsion were prepared, and 0.30 cc. of each were mixed with an equal volume of the serum to be tested. After incubation at room temperature for 1 hour or longer, 0.25 cc. of each mixture were inoculated intradermally in a rabbit. In order to avoid the individual variations in susceptibility of rabbits to vaccinal infections, in so far as possible all samples of serum from one experimental animal were tested on a single rabbit at the same time. By this means, consistent results in regard to changes in the neutralizing capacity of the serum of each animal were obtained.

Tests of Resistance to Vaccinia.—After completion of a course of injections and collections of serum, the degree of immunity of each rabbit was tested by the dermal and intradermal inoculation of tenfold dilutions of vaccine virus and by the intratesticular injection of 1 cc. of a 1 :1000 dilution of the active agent. 3 strains of virus were used. The first had been carried for some time in culture and was of relatively low pathogenicity for the rabbit; the second was a testicular strain, highly virulent; the third was the strain used in preparing the elementary body suspensions, and was used in the form of a suspension of active elementary bodies. These strains of virus are designated as "culture," "BH," and "C.L.," respectively.

EXPERIMENTAL

It has been reported by Craigie (33), who worked under properly controlled conditions, that agglutinins, precipitins, and complement-fixing antibodies appeared in the blood of rabbits after inoculation with elementary bodies of vaccinia inactivated by formaldehyde or heat. We have extended this work to include a study of the response of rabbits to inactive elementary bodies as evidenced by the neutralizing capacity of their sera and resistance to infection with active virus. It has also been shown (31) that extracts of vaccinal infected tissues contain soluble substances reacting specifically with antivaccinal serum. We have studied the antigenicity of such extracts after removal of active virus from them by filtration through collodion membranes.

In investigations concerning the response of animals to inoculation with noninfectious preparations of vaccine virus, it is desirable that suspensions of the active agent be as free as possible from other particulate or soluble materials in order that the action of virucidal agents may be regular and uniform. Furthermore, it is desirable that the virus in the preparations be susceptible of concentration, in order that a representative sample may be tested for activity of the treated agent. A suspension of elementary bodies of vaccinia satisfies these requirements. Soluble materials and particulate matter different from the elementary bodies can be removed by repeated washing in dilute buffer and differential centrifugation, and the virus in the final preparations can be concentrated to any desired extent by means of the angle centrifuge. Moreover, in such investigations care must be taken that rabbits do not become accidentally infected with vaccinia. Consequently, throughout this work proper isolation precautions were observed.

Experiment 1

Before undertaking extensive studies to determine the response of rabbits to injections of noninfectious formolized elementary bodies of vaccinia and to virus-free extracts of dermal vaccine virus, it seemed best to carry out one or two orientation experiments.

4 rabbits were used from which samples of serum were collected before the injections were begun. 2 were inoculated with a suspension of elementary bodies

inactivated by the addition of 0.3 per cent formaldehyde, and two received injections of an extract of dermal vaccine virus freed from active virus by filtration through collodion membranes. Inoculations were made weekly over a period of 6 weeks. 15 cc. of material were given each animal. At first, injections were made intravenously, but, after the third administration of the filtrate, one of the rabbits died in convulsions within a few minutes. The remaining rabbits, therefore, were subsequently injected intraperitoneally. Blood was taken again from each animal 1 week after the last injection, and the rabbits were tested for immunity to active virus after another week. In this experiment a suspension of active elementary bodies was used in the test of resistance.

TABLE I

Results of Inoculation of Rabbits with Formolized (0.3 Per Cent) Elementary Bodies and Virus-Free Filtrate of Dermal Vaccine Virus Extract

Rabbit No.	Serum*			Inocula	Serum*				Virus inoculation†	
	Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate		Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate	Neutralization	Strain	Titer—Intradermal
8-37	—	—	—	Formolized elementary bodies	32	2	8	10^2, 10^3, 10^4	C.L.‡	10^7
8-38	—	—	—	" "	32	4	16		C.L.	10^5
8-39	—	—	—	Dermal filtrate			Died			
8-40	—	—	—	" "	8	4	4	1, 10, 10^2	C.L.	10^5
9-10				None					C.L.	10^7

* Figures indicate highest dilution of serum giving perceptible agglutination or precipitation, or the number of "infectious doses" of vaccine virus neutralized by an equal volume of serum. Neutralization tests were performed on different rabbits and results, therefore, are irregular.

† Figures indicate highest dilution of virus giving perceptible lesions.

‡ C. L. signifies Connaught Laboratory strain of vaccine virus, propagated on scarified skin.

The results of the experiment are summarized in Table I. None of the rabbits possessed agglutinins or precipitins for vaccine virus prior to the inoculations. Afterward, these antibodies were present in all. The titer of agglutinins ranged from 1:8 to 1:32; that of precipitins from 1:2 to 1:16. Neutralization experiments were carried out only on samples of serum obtained after completion of the course of re-

peated inoculations. The pooled sera of the 2 rabbits inoculated with inactive elementary bodies neutralized 10^2 to 10^1 infectious units of virus, while the serum of the animals that received virus-free extracts neutralized from 1 to 10^2 units. Although these tests were done on different rabbits at different times and the neutralization titers are not comparable, yet it is evident that the sera were capable of neutralizing moderate amounts of active virus. It is noteworthy that the animals inoculated with inactive elementary bodies possessed more humoral antibodies than did the one that received the virus-free filtrate. When tested for resistance to vaccinia by means of intradermal inoculations of active elementary bodies, the treated and control rabbits responded in a similar manner with the exception that the former developed considerable edema at the sites of inoculations shortly after the injections were made. The edema disappeared within 48 to 72 hours, after which the lesions and the titers of the virus in all of the animals were not strikingly different.

Experiment 2

In the previous experiment it was shown that agglutinating, precipitating, and virus-neutralizing antibodies appeared in the serum of rabbits inoculated repeatedly with small doses of preparations of noninfectious elementary bodies, while resistance to infection with a strong virus was not appreciably altered. It then seemed advisable to learn whether a single inoculation of a large quantity of inactive virus would be more effective.

In order to give at one time a quantity of virus similar to that previously used in repeated doses, it was desirable to reduce the volume in which it was contained. This was done as follows: A suspension of elementary bodies was prepared in the usual manner to which 0.3 per cent of formaldehyde was added. After remaining in contact with the formaldehyde for 2 weeks the elementary bodies were sedimented in the angle centrifuge and taken up in a small amount of Locke's solution. Each 1.5 cc. of the concentrated material represented the amount of virus contained in 20 cc. of the original preparation, while the concentration of formaldehyde was greatly reduced. Samples of serum were taken from two rabbits and then each animal received 1.5 cc. of the concentrated inactive virus, 0.5 cc. intradermally and 1.0 cc. intratesticularly. In neither animal did local reactions and fever occur. 2 weeks later blood was taken again for the study of humoral antibodies, after which tests for resistance to culture and BH viruses were conducted.

The results of the above experiment are set forth in Table II. Before treatment the rabbits possessed no demonstrable humoral antibodies for vaccine virus. Afterwards, however, agglutinin and precipitin titers were 1:256 and 1:2 or zero, respectively, and the sera neutralized 1 infectious dose of virus. Control and treated rabbits responded in the same manner to inoculations of BH and culture viruses. Samples of serum collected from the treated and control

TABLE II

Results of Inoculation of Rabbits with Single Massive Injections of Formolized (0.3 Per Cent) Elementary Bodies Injected Intradermally and Intratesticularly

Rabbit No.	Serum*				Inocula	Serum*				Virus inoculation†						Serum*			
	Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate	Neutralization		Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate	Neutralization	Strain	Titer—intradermal	Strain	Titer—intradermal	Temperature	Testicular reaction	Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate	Neutralization
														°F.					
5-76	—	—	—	—	Formolized elementary bodies	256	2	2	1	C‡	10^4	BH§	10^6	105.7	+	1024	16	16	10^5
5-78	—	—	—	—	" "	256	2	—	1	C	10^5	BH	10^6	105.5	+	512	16	32	10^5
5-75	—	—	—	—	None	—	—	—	—	C	10^4	BH	10^6	105.3	+	512	2	1	10^5
5-77	—	—	—	—	"	—	—	—	—	C	10^4	BH	10^6	105.6	+	512	4	4	10^5
5-79	—	—	—	—	"	—	—	—	—	C	10^4	BH	10^6	105.4	+	1024	2	1	10^5

* Figures indicate highest dilution of serum giving perceptible agglutination or precipitation, or the number of "infectious doses" of vaccine virus neutralized by an equal volume of serum.

† Figures indicate highest dilution of virus giving perceptible lesions.

‡ C indicates culture vaccine virus.

§ BH indicates New York City Board of Health vaccine virus, propagated in testicles of rabbits.

animals after the test for resistance to active virus neutralized 10^5 infectious doses of virus and possessed agglutinin titers of 1:512 to 1:1024 and precipitin titers of 1:1 to 1:32. It is interesting to note that there was a marked difference between the precipitin titers, 1:16 –1:32, of serum from previously treated rabbits and the titers, 1:1–1:4, of serum from the controls that had not had any inoculations prior to the injections of active virus. This fact seems to indicate

that some change had occurred in the rabbits as a consequence of the inoculation of inactive elementary bodies although it was not evident in the tests for resistance to infection with active virus.

From the results of this experiment it is obvious that the administration of the inactive virus in 1 large dose is no more effective than repeated injections of small doses. In fact, in view of the results obtained in subsequent experiments it appears that the former method is less effective than the latter.

Experiment 3

Since it appeared from the results of the experiments just described that the rabbits showed a certain amount of response to injections of inactive elementary bodies, we decided to carry out more extensive and better controlled experiments in which the animals after receiving repeated inoculations of inactive elementary bodies or virus-free extracts of dermal vaccine virus were tested for resistance to infection with a weak virus as well as with a very potent one.

10 rabbits were used. Of these, 3 received repeated inoculations of a suspension of elementary bodies inactivated by addition of 0.3 per cent formaldehyde, 3 received virus-free extract of dermal virus, 2 control rabbits were injected with meat infusion broth and 2 with typhoid vaccine. The materials were prepared, and the inoculations were made over a period of 6 weeks as described above under Methods and Materials. 2 weeks after the last set of injections the rabbits were tested for resistance to 2 kinds of active virus administered dermally, intradermally, and intratesticularly. A second inoculation of the potent virus was given 2 weeks later. Each time the treated rabbits were inoculated with active virus, normal control rabbits also received similar inoculations. Serum was collected from each rabbit before treatment and at different times during the experiment for the study of humoral antibodies.

When one examines the results of Experiment 3, summarized in Table III, it is immediately obvious that none of the rabbits possessed humoral antibodies against vaccine virus at the beginning of the experiment and that the repeated injections of meat infusion broth or of typhoid vaccine did not produce antiviral antibodies or resistance to vaccinia in the animals receiving them. The rabbits that were treated with formolized elementary bodies or with virus-free extracts of dermal virus developed fair agglutinin and precipitin titers ranging

TABLE III

Results of Inoculation of Rabbits with Elementary Bodies, Inactivated by Means of 0.3 Per Cent Formaldehyde, and with Virus-Free Filtrates of Dermal Vaccine Virus

Rabbit No.	Serum*			Inocula	Serum*			Virus inoculation†					Serum*			Virus reinoculation†		Serum*		
	Agglutination	Precipitation with testicle filtrate	Neutralization‡		Agglutination	Precipitation with testicle filtrate	Neutralization‡	Strain	Titer—intradermal	Titer—scarification	Testicular reaction	Maximum temperature	Agglutination	Precipitation with testicle filtrate	Neutralization‡	Strain	Titer—intradermal	Agglutination	Precipitation with testicle filtrate	Neutralization‡
												°F.								
0.39	—	—	—(—)	Elementary bodies	512	16	10(10)	C	0	0	0	103.4	512	16	10(10)	BH	10^5	256	16	(10^3)
0.40	—	—	—(—)	" "	256	8	10^2(1)	C	10^1	0	0	103.6	128	2	10^2(1)	"	10^5	128	8	(10^2)
0.41	—	—	—(—)	" "	256	16	10	BH	10^6	10^4	±	105.2	512	32	10^5	"	10^3	512	16	10^4
0.47	—	—	—(—)	Virus-free filtrate	256	16	10(10)	C	10^3	0	±	103.0	128	16	10(10)	"	10^5	128	16	(10^3)
0.50	—	—	—(—)	" "	128	8	1(0)	C	10^5	0	+	104.0	128	16	10^4(10^4)	"	10^2	128	8	(10^4)
0.51	—	—	—(—)	" "	64	16	1(1)	BH	10^6	10^4	+	101.4	Died							
0.58	—	—	—	Broth	—	—	—	C	10^6	10^2	+	105.4	256	16	10^5(10^2)	"	10^1	256	8	(10^2)
0.59	—	—	—	"	—	—	—	C	10^3	10^2	+	104.2	256	8	10^4(10^4)	"	10^1	256	16	(10^4)
0.63	—	—	—	Typhoid vaccine	—	—	—	C	10^4	10^2	+	105.2	256	8	10^3(10^3)	"	10^1	256	16	(10^3)
0.64	—	—	—	" "	—	—	—	C	10^5	10^2	+	104.4	256	8	10	"	10^2	256	8	10^4
1.99				None				C	10^5	10^2	+	105.1	64	4	10^5	"	10	64	4	10^5
2.00				"				C	10^5	10^2	+	105.1	64	16	10^6	"	10	64	16	10^6
2.01				"				C	10^5	10^2	+	105.8	256	16	10^4	"	10^2	128	16	
2.02				"				C	10^5	10^2	+	105.8	128	8	10^5	"	10	256	8	
2.69				"												"	10^0	512	8	10^5
2.25				"				BH	10^6	10^5	+	107.0	Died							

* Figures indicate highest dilution of serum giving perceptible agglutination or precipitation, or the number of "infectious doses" of vaccine virus neutralized by an equal volume of serum.

† Figures indicate highest dilution of virus giving perceptible lesions.

‡ All samples of serum from a single animal were tested on the same rabbit. Results of duplicate tests, made on another rabbit, are bracketed in order to facilitate comparison of the results.

from 1:64 to 1:512 and from 1:8 to 1:16, respectively. The neutralizing titers of the same sera, however, were very low, inactivating only 1 to 10 infectious doses of active virus. It is true that one of these sera (0-40) inactivated 100 doses of virus in one test, but when investigated again it neutralized only 1 dose.

One of the 3 rabbits that had been treated with inactive elementary bodies showed no evidence of infection when inoculated dermally, intradermally, and intratesticularly with culture virus, an agent of weak pathogenicity for rabbits. Another animal of this group similarly inoculated showed a vaccinal lesion only at the point where the lowest dilution of the culture virus was introduced intradermally. The third animal of the group received dermal, intradermal, and intratesticular inoculations of BH virus, an agent of strong pathogenicity for the rabbit, and showed only a slight amount of resistance to infection. In fact, it was necessary to compare the lesions in the treated rabbit with those in a control in order to appreciate that the inoculations of inactive elementary bodies had modified the course of the infection.

Of the 3 rabbits treated with virus-free extracts, 2 were tested with active culture virus and 1 with the BH virus and evidenced much less resistance than did those that had been treated with inactive elementary bodies. No lesions occurred at the sites of dermal inoculation of culture virus. Lesions did occur, however, as the result of intratesticular and intradermal injections of the active agent, and one of the animals had fever. Although 10^3 and 10^5 dilutions of the virus produced lesions when injected intradermally such lesions were definitely different from those that occurred in controls. The rabbit that was tested with the BH virus showed no evidence of having been benefited by the treatments with virus-free extracts; in fact, it died of the vaccinal infection.

Samples of serum collected from treated and control rabbits 2 weeks after the first inoculations of active virus gave interesting results when tested for humoral antibodies. All of the control animals showed good titers of agglutinins, precipitins, and neutralizing antibodies. Rabbit 0-41 that received active BH virus after treatment with inactive elementary bodies and developed a definite infection, showed a rise in the amount of all humoral antibodies, and animal

0-50 that received active culture virus after treatments with virus-free extracts and developed an infection, also showed a marked rise in its neutralizing antibodies. The other treated rabbits (0-39, 0-40, 0-47) after inoculations of the active culture virus showed either no change or a decrease in the amounts of agglutinins, precipitins, and neutralizing antibodies.

In order to determine whether the findings just described were due to defects in the methods of testing the humoral antibodies or represented a failure of the weak culture virus to enhance a slight amount of immunity already present, the animals were reinoculated with the potent BH virus. The rabbits that had responded to the first inoculations of active virus with evident infections manifested on this occasion a fairly high degree of resistance with lesions only at the sites of inoculation of 1:100 and 1:1000 dilutions of the virus and no increase in their titers of neutralizing antibodies. On the other hand, the treated rabbits, previously refractory to active weak culture virus, developed lesions at the sites of inoculation of 1:100,000 dilutions of the BH virus; necrotic areas appeared in the center of the lesions caused by undiluted virus and that diluted ten times; and there was a sharp rise in the neutralizing titers of the sera. In comparing the neutralization titers of samples of serum from rabbit 0-40 only the bracketed figures should be considered at this time.

When one examines the results (Table III) obtained by inoculating normal rabbits with culture virus and compares them with the ones just described it seems that a weak immunity induced by inactive virus or virus-free material can at times interfere or prevent an enhancement of this slight amount of resistance by means of a weak active agent.

Experiment 4

In order to determine whether an increase in the length of time that the elementary bodies remained in contact with the 0.3 per cent formaldehyde or whether a moderate increase in the concentration of formaldehyde used for inactivation of the virus would yield results different from those obtained in Experiment 3, another experiment was carried out in the following manner.

TABLE IV

Results of Inoculation of Rabbits with Elementary Bodies Which Had Been Suspended in 0.3 Per Cent Formaldehyde for 3½ Months and 1.0 Per Cent Formaldehyde for an Additional 10 Days

Rabbit No.	Serum*				Inocula	Serum*				Virus inoculation†					Serum*				Virus reinoculation†				Serum*			
	Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate	Neutralization		Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate	Neutralization	Strain	Titer—intradermal	Titer—scarification	Testicular reaction	Maximum temperature	Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate	Neutralization	Strain	Titer—intradermal	Testicular reaction	Maximum temperature	Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate	Neutralization
														°F.							°F.				
2-50	—	—	—	—	Elementary bodies 0.3% formaldehyde	256	4	4	10^1	C	10^1	—	—	103.1	128	—	—	10^2	BH	10^1(E)	—	103.8	128	4	8	10^5
2-51	—	—	—	—	" "	256	4	8	10^5	BH	10^3	10^1	±	103.1	256	8	16	10^5	BH	10^1(E)	—	102.9	128	8	16	10^5
2-52	—	—	—	—	Elementary bodies 1.0% formaldehyde	128	1	4	10^1	C	10^2	10^1	—	103.0	64	—	2	10^4	BH	10^1(E)	—	102.9	128	4	32	10^5
2-53	—	—	—	—	" "	256	4	16	1	BH	10^5	10^2	—	103.2	256	4	16	10^4	BH	10^3(E)	—	‡	256	16	32	10^5
2-56	—	—	—	—	Broth	—	—	—	—	C	10^5	10^2	+	103.6	128	4	8	10^5	BH	10^1	±	103.2	128	8	16	10^5
2-54	—	—	—	—	"	128	4	8	10^4	BH	10^1	10^1	—	102.4	64	2	8	10^4	BH	10^2	±	103.2	256	32	16	10^5
2-76	—	—	—	—	None	—	—	—	—	C	10^5	10^2	+	105.2	256	2	8	10^5	BH	10^2	+	103.4	128	8	16	10^5
2-57	—	—	—	—	"	—	—	—	—	BH	10^5	10^1	+	106.6	256	8	32	10^5	BH	10^1	—	103.0	512	32	32	10^5
4-59					"					C	10^2	10^2	+	105.0	128	2	16	10^3	BH	10^1(E)	—	102.5				10^5
4-58					"					BH	10^5	10^3	+	105.4	Died											
5-10					"														BH	10^5	+	104.2				

(E), marked edema at site of injection, subsiding in 48 to 72 hours.

* Figures indicate highest dilution of serum giving perceptible agglutination or precipitation, or the number of "infectious doses" of vaccine virus neutralized by an equal volume of serum.

† Figures indicate highest dilution of virus giving perceptible lesions.

‡ Animal developed snuffles with fever; temperature readings, therefore, are not significant.

A portion of the elementary body suspension prepared for Experiment 3 was employed; the virus had been in contact with 0.3 per cent formaldehyde for a period of over 3 months. The material was divided into 2 portions. To one of them sufficient formaldehyde was added to increase the final concentration from 0.3 to 1.0 per cent. After a period of 10 days the suspensions were used for the repeated intraperitoneal inoculation of rabbits according to the schedule used in the previous experiment. 2 rabbits were used for each of the preparations of elementary bodies, 2 rabbits received meat infusion broth, and 2 rabbits received no injections. Samples of serum were collected and studied, and the animals were tested twice for immunity to active virus as in Experiment 3.

An examination of the results of the above experiment, summarized in Table IV, reveals that they are similar to those recorded for Experiment 3 and indicate that contact of elementary bodies of vaccinia with 0.3 per cent formaldehyde for $3\frac{1}{2}$ months or contact with 0.3 per cent formaldehyde for over 3 months followed by contact with 1.0 per cent of it for 10 days does not completely destroy their antigenicity. Inasmuch as the results are similar to those of Experiment 3, a detailed discussion of them will be omitted.

Two points of special interest are found in Table IV. 5 of the rabbits, Nos. 2-50, 2-51, 2-52, 2-53, and 4-59, rapidly developed a marked edema, which subsided within 48 to 72 hours, at the sites of the second set of inoculations of active virus. After the disappearance of the edema, the vaccinal lesions seemed to proceed in the usual manner. Inasmuch as antiviral antibodies appeared in its serum during the time that only injections of broth were being administered, it is obvious that rabbit 2-54 became accidentally infected with vaccinia in spite of precautions taken to prevent such an occurrence. Moreover, the high neutralizing titer (10^5) of the serum of rabbit 2-51 that received elementary bodies inactivated by 0.3 per cent formaldehyde and the relatively good resistance to potent BH virus subsequently evidenced by the animal, lead one to suspect that an accidental infection with active virus also occurred in this rabbit.

Experiment 5

The experiments described above demonstrated that moderate amounts of formaldehyde decrease but do not completely destroy the antigenicity of the elementary bodies of vaccinia. It seemed desirable therefore, to ascertain whether more drastic treatment with formaldehyde or heat is capable of effecting such a destruction.

A suspension of highly purified elementary bodies was prepared and divided into 2 portions. One portion was boiled for 2 hours. The elementary bodies in

the other portion were sedimented by centrifugation and resuspended in a small amount of 10 per cent formaldehyde. After 10 days the formolized suspension was diluted with 10 times its volume of buffer solution. 2 rabbits received repeated intraperitoneal inoculations of the formolized elementary bodies, 2 were inoculated with the boiled elementary bodies, and 2 that were given virus-free extracts prepared from emulsions of tissues containing herpetic virus served as controls. Inasmuch as the suspension of elementary bodies was more concentrated

TABLE V

Results of Inoculation of Rabbits with Elementary Bodies Inactivated by Means of 10 Per Cent Formaldehyde or by Boiling

Rabbit No.	Serum*			Inocula	Serum*			Virus inoculation†					Serum*		
	Agglutination	Precipitation with testicle filtrate	Neutralization		Agglutination	Precipitation with testicle filtrate	Neutralization	Strain	Titer—intradermal	Titer—scarification	Testicular reaction	Maximum temperature	Agglutination	Precipitation with testicle filtrate	Neutralization
												°F.			
3-44	—	—	—	Formolized elementary bodies	32	—	—	BH	10^4	10^2	—	104.5	256	16	10^6
3-45	—	—	—	" "	2	—	—	C	10^3	10^2	±	102.8	128	4	10^3
3-46	—	—	—	Heated elementary bodies	2	—	—	BH	10^4	10^4	+	106.0	128	16	10^6
3-47	—	—	—	" "	8	—	—	C	10^3	10^1	—	103.8	64	4	10^3
3-54	—	—	—	Herpes virus extract plus swine serum	—	—	—	BH	10^5	10^5	+	105.4	256	8	10^6
3-56	—	—	—	" "	—	—	—	C	10^1	10^1	—	‡	2	2	10^6
5-00				None				BH	10^4	10^4	+	105.8	512	16	10^4
5-02				"				C	10^3	10^1	+	102.8	64	—	10^2
5-01				"				BH	10^5	10^5	+	106.4	256	16	10^4
5-03				"				C	10^3	10^2	—	101.8	32	1	10^3

* Figures indicate highest dilution of serum giving perceptible agglutination or precipitation, or the number of "infectious doses" of vaccine virus neutralized by an equal volume of serum.

† Figures indicate highest dilution of virus giving perceptible lesions.

‡ Animal developed snuffles; temperature determinations, therefore, are not significant.

than those previously used, each rabbit received only 12 cc. of material given in 6 doses over a period of 3 weeks. Serum was collected from all the rabbits at the beginning of the experiment and at different times during it. 2 weeks after completion of the series of injections the treated rabbits and normal controls were tested for resistance to active BH and culture viruses.

The results of Experiment 5, summarized in Table V, show that repeated inoculations of elementary bodies that had been boiled or

treated with 10 per cent formaldehyde did not induce in rabbits the production of precipitins, neutralizing antibodies, or resistance to active vaccine virus. However, a few agglutinins, titers ranging from 1:2 to 1:32, did appear in the sera of the inoculated rabbits.

Experiment 6

It has been stated (34) that virus-free extracts of testicular vaccine virus although containing substances precipitable by antivaccinal serum, do not induce the production in rabbits of antiviral antibodies. In Experiments 1 and 3, however, we found that animals repeatedly inoculated with virus-free extracts of dermal vaccine virus developed humoral antibodies and in certain instances a slight amount of resistance to infection with vaccinia. Inasmuch as some workers have suggested (34) that the precipitinogen in these extracts is a haptene, it occurred to us that perhaps an incomplete antigen or haptene in the dermal extracts was completed by the foreign protein liberated by the bacteria inevitably present in dermal virus. We tested this idea by using as inocula virus-free extracts containing the precipitinogen or precipitinogens prepared from testicular vaccine virus free from bacterial contaminants, and such extracts to which sterile swine serum had been added. Virus-free extracts of testicular herpes virus plus swine serum were used as control inocula.

An emulsion of testicular vaccine virus was prepared by grinding dried infected testicular tissue with Locke's solution; 100 cc. of fluid were used for each gram of dried material. After centrifugation the emulsion was passed through a Seitz filter and then a collodion membrane. The filtrate was shown to be virus-free. The material was divided into 2 portions. One portion was used for inoculation without further treatment. To the other was added sufficient sterile swine serum to make its concentration 10 per cent. As a control a similar preparation of herpes testicular virus was made to which pig serum was added. 3 rabbits received the vaccine virus extracts alone, 2 the vaccine virus extracts plus swine serum, and 3 the herpes virus extracts plus swine serum. Each rabbit received 12 cc. of material administered intraperitoneally in 6 doses over a period of 3 weeks. Samples of serum were collected at different times for the study of humoral antibodies. 2 weeks after the last inoculations, the animals were tested for resistance to infection with vaccinia, both culture and BH strains of virus being used.

The results of Experiment 6, summarized in Table VI, show that the animals receiving the extract of testicular herpes virus plus swine

serum developed no antivaccinal antibodies, while those that were given extracts of testicular vaccine virus or extracts of testicular vaccine virus plus swine serum only responded with the production of slight amounts—in some instances none—of agglutinins, precipitins, and protective or neutralizing antibodies. Furthermore, it is obvious

TABLE VI

Results of Inoculation of Rabbits with Virus-Free Filtrate of Testicular Vaccine Virus Extract and Virus-Free Filtrate of Testicular Virus Extract Plus Swine Serum

Rabbit No.	Serum*				Inocula	Serum*				Virus inoculation†					Serum*			
	Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate	Neutralization		Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate	Neutralization	Strain	Titer—intradermal	Titer—scarification	Testicular reaction	Maximum temperature	Agglutination	Precipitation with dermal filtrate	Precipitation with testicle filtrate	Neutralization
														°F.				
3-48	—	—	—	—	Testicle virus extract	8	—	—	—	BH	10^5	10^3	+	104.0	128	16	32	10^5
3-49	—	—	—	—	" "	8	—	2	—	BH	10^4	10^4	+	107.0		Died		
3-50	—	—	—	—	" "	256	2	8	—	C	10^3	10^1	+	102.3	128	16	16	10^1
3-51	—	—	—	—	Testicle virus extract plus swine serum	8	—	—	—	BH	10^4	10^3	+	105.8		Died		
3-52	—	—	—	—	" "	—	—	—	1	C	10^3	10^1	+	107.2	—	4	2	10^5
3-54	—	—	—	—	Herpes virus extract plus swine serum	—	—	—	—	BH	10^5	10^5	+	105.4	256	8	8	10^6
3-55	—	—	—	—	" "	—	—	—	—	BH	10^4	10^4	+	105.0		Died		
3-56	—	—	—	—	" "	—	—	—	—	C	10^1	10^1	—	‡	2	—	2	10^6
5-00					None					BH	10^4	10^4	+	105.8	512	8	16	10^4
5-01					"					BH	10^5	10^5	+	106.4	256	4	16	10^4
5-02					"					C	10^3	10^1	+	102.8	64	2	—	10^2
5-03					"					C	10^3	10^2	—	101.8	32	2	1	10^3

* Figures indicate highest dilution of serum giving perceptible agglutination or precipitation, or the number of "infectious doses" of vaccine virus neutralized by an equal volume of serum.

† Figures indicate highest dilution of virus giving perceptible lesions.

‡ Animal developed snuffles; temperature determinations are, therefore, not significant.

that the addition of swine serum to the extract of testicular vaccine virus did not make the latter material a better antigen for the stimulation of antivaccinal antibodies. None of the rabbits evidenced, as a result of the repeated inoculations, an appreciable degree of resistance to infection with vaccinia; in fact, 3 of them died. Accurate

readings of the reactions were difficult because of the marked spreading of the infection around the sites of inoculation which doubtless occurred in consequence of the rabbits having received large amounts of material containing the "spreading factor" of Duran-Reynals (35).

In Experiments 1 and 3 the rabbits that received inoculations of virus-free extracts of dermal vaccine virus produced more humoral antibodies and evidenced more resistance, even though it was not a great amount, than did the animals in Experiment 6 that were given injections of virus-free extracts of testicular vaccine virus. This discrepancy in the results of the two sets of experiments may be accounted for in two ways. Firstly, in Experiments 1 and 3 each rabbit received 15 and 18 cc. of dermal extracts, respectively, administered in 12 doses over a period of 6 weeks, while in Experiment 6 each rabbit received only 12 cc. of testicular extract administered in 6 doses over a period of 3 weeks. Secondly, according to our findings, the concentration of the precipitable vaccinal substances or antigens in the dermal extracts is greater than in the testicular extracts.

Experiment 7

Having determined that humoral antibodies and a certain amount of resistance to vaccinia can be produced in rabbits by the repeated injection of inactive formolized elementary bodies and virus-free preparations of the precipitable vaccinal antigens, we decided to investigate their persistence for a comparison with the phenomena that follow a frank infection with vaccine virus. To this end the following experiment was carried out.

The rabbits were treated with inactive formolized elementary bodies of vaccinia, virus-free filtrates of dermal vaccine virus, typhoid vaccine, and meat infusion broth in a manner identical with that employed in Experiment 3 with the exception that they were held for 114 days, instead of 14, after the series of inoculations before being tested for resistance to infection with active vaccine virus. 1 rabbit received inactive elementary bodies, 1 virus-free filtrate, 1 typhoid vaccine, and 1 broth. For comparison 2 rabbits were inoculated with active BH virus and held in another room. At the beginning, during, and at the end of the experiment samples of serum were collected from each rabbit for the estimation of humoral antibodies.

TABLE VII

Duration of Immunity in Rabbits Following Repeated Inoculations of Virus-Free Extracts of Dermal Vaccine Virus and Elementary Bodies Inactivated by Means of 0.3 Per Cent Formaldehyde

Rabbit No.	Serum* before inoculation: Agglutination	Serum* before inoculation: Precipitation with testicle filtrate	Serum* before inoculation: Neutralization	Inocula	Serum* after 14 days: Agglutination	Serum* after 14 days: Precipitation with testicle filtrate	Serum* after 14 days: Neutralization	Serum* after 78 days: Agglutination	Serum* after 78 days: Precipitation with testicle filtrate	Serum* after 102 days: Agglutination	Serum* after 102 days: Precipitation with testicle filtrate	Serum* after 114 days: Agglutination	Serum* after 114 days: Precipitation with testicle filtrate	Serum* after 114 days: Neutralization	Virus inoculation†: Strain	Virus inoculation†: Titer—intradermal	Virus inoculation†: Testicular reaction	Virus inoculation†: Maximum temperature	Virus inoculation†: Strain	Virus inoculation†: Titer—intradermal	Serum* after virus inoculation: Agglutination	Serum* after virus inoculation: Precipitation with testicle filtrate	Serum* after virus inoculation: Neutralization
																		°F.					
0-46	—	—	—	Formolized elementary bodies	256	8	10^4	32	2	8	2	16	—	10^2	BH	10^5	+	105.0	C	10^4	128	32	10^5
0-82	—	—	—,—	Virus-free filtrate	64	16	10,10	4	1	2	—	2	—	0,0	BH	10^5	+		C	10^2		Died	
0-62	—	—	—	Broth	—	—	—	—	—	—	—	—	—	—	BH	10^5	+	106.2	C	10^4	128	4	10^5
0-65	—	—	—	Typhoid vaccine	—	—	—	—	—	32	8	16	1	10^4	BH	10^2	—	103.3	C	—	16	2	10^5
2-69				Active BH virus	512	8	10^5					128	4	10^4	BH	10^1	—	103.0			128	16	10^4
2-70				" " "	512	16	10^4					256	2	10^4	BH	10^1	±	103.8			64	2	10^4
5-00				None											BH	10^4	+	105.8			512	16	10^4
5-01				"											BH	10^5	+	106.4			256	16	10^4
5-02				"															C	10^3	64	—	10^2
5-03				"															C	10^3	32	1	10^3

* Figures indicate highest dilution of serum giving perceptible agglutination or precipitation, or the number of "infectious doses" of vaccine virus neutralized by an equal volume of serum.

† Figures indicate highest dilution of virus giving perceptible lesions.

From the results of Experiment 7, summarized in Table VII, it is at once apparent that none of the animals possessed humoral antibodies at the beginning of the experiment and that one of the control rabbits, No. 0-65, that received the typhoid vaccine was accidentally infected with active vaccine virus in spite of our efforts to prevent such an occurrence. As a result of repeated injections of inactive elementary bodies and virus-free filtrates, respectively, in rabbits 0-46 and 0-82, considerable amounts of humoral antibodies, more in the former animal than in the latter, were developed, all of which were gradually decreasing in amount when last investigated 114 days after completion of the series of injections. Both of these rabbits when inoculated with active BH and culture viruses seemed fully susceptible to infection. In the control rabbits 2-69 and 2-70 that received the BH virus at the beginning of the experiment, the agglutinins and precipitins, but not the neutralizing antibodies, also gradually decreased in amount during the detention period of 114 days. However, when these rabbits were reinoculated with active BH virus, an almost complete resistance to infection was exhibited.

It is obvious that too few rabbits were used in Experiment 7 to warrant definite conclusions. At that time more animals could not be properly housed in our isolation room. Nevertheless, when one examines the results of Experiments 3 and 7, it appears that the slight amount of resistance to vaccinal infection produced in rabbits by repeated injections of inactive elementary bodies or virus-free filtrates of dermal virus tends to disappear rapidly, while that caused by active virus is more enduring.

DISCUSSION

In the study of infectious diseases the importance of an accurate knowledge of the response of animals to the inciting agents in an inactive as well as active state has long been recognized. Such information leads to a better insight into the mechanism of immunity in which multiplicity, lability, and stability of antigens, phenomena of infection, and intimacy, duration, and possibly persistence of infection play important rôles. With this increase of knowledge regarding immunity one may reasonably expect the appearance of opportunities to prevent certain diseases by means of vaccines made of inactive

or attenuated agents or to improve methods of vaccination already in use.

As pointed out earlier in the paper many investigations along the lines referred to have been made with vaccine virus, but, as indicated, practically all of the work is subject to criticism for one or more reasons. Recent advances in the knowledge of vaccine virus have made it possible for us to eliminate certain of the sources of error by the use of preparations of highly purified elementary bodies and virus-free extracts of virus-infected tissues containing specific vaccinal antigens. Great care was taken to be as certain as now is possible that no active virus remained in the preparations spoken of as inactive. Furthermore, in view of the ease with which rabbits are accidentally infected with active vaccine virus, rigid isolation precautions were observed. In spite of such precautions, 2 control rabbits and probably 1 test animal were accidentally infected with vaccinia. It is obvious, however, from the results shown in the seven tables that these accidental infections do not invalidate the conclusions we have drawn. Nevertheless, they emphasize the fact that any work of this nature conducted with inactive vaccine virus in the absence of rigid isolation is valueless.

It is apparent from the data presented that the repeated injections in rabbits of large quantities of elementary bodies of vaccinia inactivated by small amounts of formaldehyde (0.3 per cent) led to the appearance in the serum of specific agglutinins, precipitins, and neutralizing antibodies as well as to the development in the animals of a certain degree of resistance to infection with vaccinia. The immunity thus induced was slight and apparently not enduring, and was best demonstrated by the use of a strain of virus of low pathogenicity for the rabbit. A similar but less marked response followed the repeated injections of virus-free extracts of dermal vaccine virus. Furthermore, the inactive elementary bodies and virus-free extracts were less efficient antigens when similar amounts were given in single large doses instead of repeated small ones. Finally, drastic treatment (10 per cent formaldehyde or boiling for 2 hours) almost completely altered or destroyed the antigenicity of elementary bodies.

In a certain number of the rabbits that had received repeated injections of inactive elementary bodies or virus-free extracts (Experi-

ment 3) little or no apparent infection resulted from dermal, intradermal, and intratesticular inoculations of active culture virus. Nor was there an appreciable increase in the amount of neutralizing antibodies in the serum of the animals after they had received this active virus. 2 weeks after this test for resistance to infection with culture virus, another test was made with the highly pathogenic BH virus, at which time the rabbits were found still to be moderately susceptible to vaccinia. These results are different from those obtained with normal rabbits in which a good resistance to the BH strain of virus is developed as a result of inoculations of active culture virus. The inhibition phenomenon just described did not occur regularly and the mechanism of its production is not known.

Recently, Craigie (36) has reported that there are at least two antigens in vaccine virus, one labile (L), the other stable (S). According to him, both antigens incite the production of agglutinins and precipitins. Furthermore, he suggests that the L antigen may function in the production of resistance to vaccinal infection. In view of the description of his methods, it is not unlikely that we destroyed all or most of the L antigen in our elementary bodies by the process of inactivation. In any event, our preparations seemed to have caused no infection, and with them we obtained considerable amounts of humoral antibodies, including neutralizing ones, and some resistance to infection. Such results are not surprising because as yet there is no reason (37) to suppose that vaccine virus does not contain protein, and the injection of enough foreign protein into a rabbit should induce the animal to respond in some manner. It remains to be seen, however, whether with methods of inactivation less drastic than those used by us a truly noninfectious virus can be obtained the repeated injections of which will cause a greater degree of resistance to infection.

We found no evidence in our data to suppose that agglutinins and precipitins for vaccine virus are different antibodies. Our results have been interpreted as showing that agglutination and precipitation are due to the same antibody, or, if not, to different antibodies the production of which was not independently stimulated. On the other hand we observed that the agglutinating and precipitating activity of the sera did not necessarily parallel their virus neutralizing

properties; a high titer of agglutinins and precipitins was at times associated in the same serum with a slight amount of neutralizing antibodies and *vice versa*. Furthermore, the presence of considerable amounts of neutralizing antibodies could not be taken as an indication that the animals were protected against infection.

If our elementary bodies were really inactive—there is no reason to suppose that they were not—, then the neutralizing antibodies resulting from the repeated injections of them into rabbits could not have been induced by an antigen produced by the hosts as a result of a vaccinal infection (38). One might suppose that such an antigen, having arisen in the animals from which the elementary bodies were obtained, had not been completely removed from the elementary bodies by repeated washing and was still operative. All one can say to such a suggestion is that the virus-free filtrates of the material from which the elementary bodies were obtained and which should have contained large quantities of the hypothetical antigen engendered by the host as a result of infection were considerably less effective in inciting the production of neutralizing antibodies than were the washed elementary bodies.

Although the results of our experiments show that a certain amount of resistance to vaccinia, probably not enduring, can be secured in rabbits by the use of inactive elementary bodies, there is still no reason to suppose that the use of such materials is suitable for the protection of human beings against smallpox. Each rabbit that developed resistance to vaccinia received all of the elementary bodies, suspended in 12 to 18 cc. of fluid, that were obtained from another rabbit infected over a large area of skin (32). Consequently, if the same amount of inactive elementary bodies per body weight were required to induce the same amount of resistance in man as in the rabbit, several hundred cubic centimeters of a very expensive vaccine would be required. And then we would have no assurance that the vaccinated individuals would be protected against smallpox.

SUMMARY AND CONCLUSIONS

Humoral antibodies and a certain degree of resistance to infection with vaccinia, probably not enduring, are produced in rabbits by the repeated injections of inactive formolized (0.3 per cent) elementary

bodies of vaccinia and virus-free filtrates of dermal vaccine virus. Single injections of large amounts of elementary bodies are not as effective as similar amounts administered in small repeated doses. Drastic treatment (10 per cent formaldehyde or boiling for 2 hours) almost completely alters or destroys the antigenicity of elementary bodies.

It appears that the production of precipitins and agglutinins does not parallel that of neutralizing antibodies and that the mere presence of such antibodies in the serum of a rabbit as the result of injections of inactive elementary bodies does not necessarily indicate that the animal possesses a great degree of resistance to infection with a potent vaccine virus.

The fact that some neutralizing antibodies appeared in the sera of rabbits that had received injections of inactive elementary bodies can be interpreted as indicating that at least not all neutralizing antibodies for vaccine virus are the result of a reaction to an antigen produced by the host in consequence of a vaccinal infection.

No evidence was obtained to show that elementary bodies inactivated by our methods (0.3 per cent formaldehyde) would serve as a suitable vaccine for the protection of human beings against smallpox.

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THE COLONY MORPHOLOGY OF TUBERCLE BACILLI

V. Influence of the pH of the Culture Medium on Colony Form*

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Plates 6 and 7

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Certain experiments recently reported have indicated that the pH of the medium on which tubercle bacilli are grown may have a profound influence on the colony morphology of these organisms. Moreover, it has been observed in this laboratory that the use of acid or alkali in isolating tubercle bacilli from infected tissue has a definite tendency to inhibit the development of certain colony variants without inhibiting the total amount of growth.

Steenken, Oatway, and Petroff (1) found that when human tubercle bacilli, Strain H-37, were grown on either Petroff's or Calmette's medium adjusted to pH 6.1, the colonies were characteristically granular and rough. By selection of colonies and repeated transplantation on these media, colony variants were obtained which possessed very low pathogenicity. However, when low, veil-like, spreading, stippled colonies were grown on Petroff's medium adjusted to pH 7.2, these variants possessed the full virulence of the parent undissociated strain. Similar observations were again reported by Steenken (2) who employed synthetic medium adjusted to pH 6.0 or pH 7.6. At pH 6.0 the growth of the human strain, H-37, was dense and compact, whereas at pH 7.6 it was veil-like and spreading. Both variants proved to be virulent for guinea pigs but animals inoculated with the variants grown at pH 7.6 died the more quickly.

Birkhaug (3) also studied certain relationships between the hydrogen ion concentration in the medium and the colony morphology of tubercle bacilli. He found maximal growth (dry weight) of S variants in acid medium, and of R variants in alkaline medium. He studied the reaction curve during growth of dissociated strains on fluid medium, observing that mammalian S forms and avian

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R forms render the medium more acid, while mammalian R forms and avian S forms cause increased alkalinity.

A short time ago we (4) observed that various strains of human tubercle bacilli in primary culture produced considerable numbers of smooth, rounded colonies, as well as flat, spreading, finely granular colonies, or others more coarsely granular and irregular in contour. In controlled experiments it was shown that greatest numbers of smooth colonies were obtained by growing the organisms on Corper's medium and by avoiding the usual procedure of destroying contaminating organisms with acid or alkali. The latter observation indicated that certain colony variants might be susceptible to acid or alkali, and therefore to differences in pH of the medium. A preliminary experiment (4) showed marked differences in colony form of organisms grown at various pH values, with a probable optimum pH for the smooth variants. The latter observation was confirmed and extended in experiments reported briefly elsewhere (5). The present communication is a detailed report of the cultural studies.

It will be shown in the following paragraphs that the colony topography in cultures of tubercle bacilli is profoundly influenced by variations in the pH of the basic medium used (Corper's). The variations to be described are not rigidly confined, but the more acid medium (pH 6.0) favors the growth of granular colonies, the more alkaline medium (pH 6.8 to 7.4) favors the growth of veil-like, spreading colony variants, whereas the greater number of smooth, glistening colonies develop on medium adjusted to pH values of 6.4 to 6.8. It will also be shown that these variations apply to strains of low virulence, as well as to strains of high virulence, and that the range of pH apparently optimum for development of smooth colonies varies with the type of organisms (human, bovine, or avian).

Materials and Methods

In order to apply the method to a study of strains of tubercle bacilli possessing widely varying pathogenic properties, twenty-five strains were used in the experiments. These included three avian, twelve bovine, and ten human type strains.[1] Data concerning the source and date of isolation of these strains are recorded in Table I. The pathogenic properties of nineteen of these strains were discussed previously (6), and for convenience are recorded in Table I.

[1] In the preliminary report it was stated that eleven bovine and eleven human type strains were used. It has since been established (6) that one of the strains of human origin was of bovine type, which accounts for the discrepancy in these statements.

TABLE I

Strains of Tubercle Bacilli Used in Cultural Studies with Data Regarding the Isolation and Pathogenic Properties of Each

Strain of T. B.	Isolated by	Isolated from	Date of isolation	Relative virulence
Avian R	M. C. Kahn*	Fowl	About 1910	Low
" S	" " "	"	" 1910	" †
" TS	Theobald Smith	"	" 1930	High
Bovine B-1	E. R. Baldwin		1904	Low
" 32	Theobald Smith	Cow	Apr., 1928	"
" 33	" "	"	May, 1928	"
" 34	" "	"	Apr., 1929	Moderate
" 35	" "	"	" 1929	Low
" 36	" "	"	" 1929	High
" 37	" "	"	Oct., 1929	Low
" 38	" "	"	Mar., 1932	High
" 39	" "	"	Oct., 1932	"
" 40	" "	"	Jan., 1933	"
" Kilty	K. C. Smithburn	Human.	Feb., 1934	Moderate
" Ravenel	M. P. Ravenel	Wrist fluid		
Human H-37	E. R. Baldwin		About 1905	High
" Jamaica	J. Freund	Human. Sputum	1905	Moderate
" MR	K. C. Smithburn	Human. Tracheal node	Autumn, 1933	"
" 3103	" " "	Human. Psoas abscess	Dec., 1933	"
" 3104	" " "	*M. rhesus.* Spleen	Jan., 1934	"
" Thompson	" " "	Human. Knee biopsy	" 1934	"
" Bell	R. M. Thomas	Human. Psoas abscess	Feb., 1934	"
" 3421	K. C. Smithburn	Human. Sputum	Reisolated Feb., 1934	"
" 3422	" " "	Human. Spinal fluid	May, 1934	"
" Burroughs	" " "	Human. Spinal fluid	" 1934	"
		Human. Sputum	June, 1934	"

* These R and S variants were supplied to us by Dr. M. C. Kahn, Cornell University Medical College, New York, on Sept. 18, 1930. They are the strains studied by Kahn (8) and Petroff (9, 10) in their respective researches on dissociation of avian tubercle bacilli.

† This variant, when obtained, was highly pathogenic but has since become attenuated without changing in colony form.

The basic medium used in the experiments was the glycerolated egg yolk medium with Congo red, proposed by Corper and Cohn (7). 3,600 cc. or 1,800 cc. lots of the medium were prepared and divided into eight or four flasks, each containing about 450 cc. The pH of each lot was determined by the glass electrode

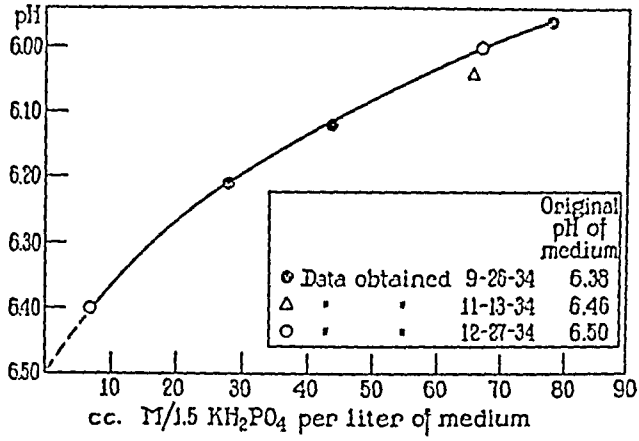

CHART 1

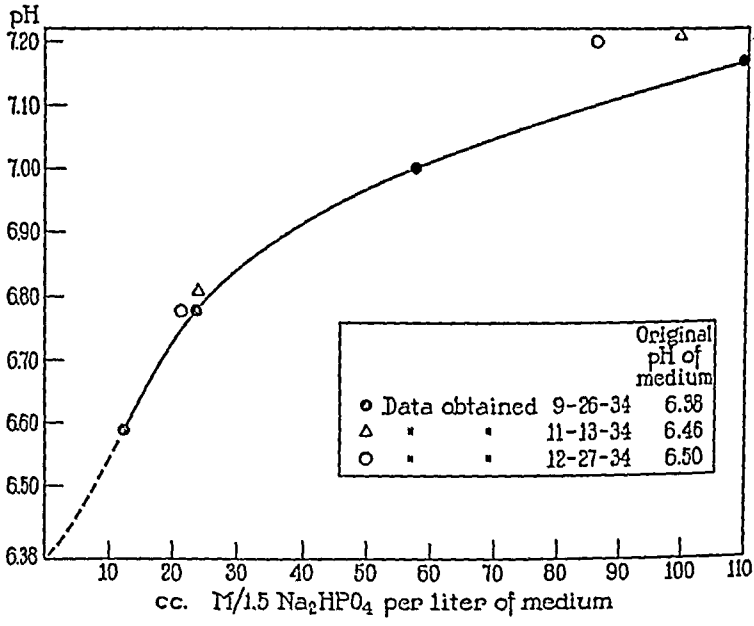

CHART 2

method.[2] The pH of each flask of medium was then adjusted to the desired value by fractional additions of M/1.5 KH_2PO_4 or Na_2HPO_4. No attempt was made to

[2] We wish to acknowledge the generosity of Drs. D. A. MacInnes, T. Shedlovsky, and Mary L. Miller, to whom we are indebted for all the pH determinations.

prepare the medium in such a manner that the pH would remain constant during growth; therefore only one buffer was added to any one lot, and this in just sufficient quantity to bring the pH to the desired value. This was a somewhat time-consuming procedure; but it was found that if a graph were plotted to show the amounts of buffer used to produce the various changes in pH, the curves obtained were quite smooth. Since various lots of standard medium varied in pH from 6.34 to 6.50, the data obtained in successive experiments did not all fall on the same curve (Charts 1 and 2), although the type of curve obtained was in each case similar.

Charts 1 and 2 are titration curves for Corper's medium against acid and alkaline buffers respectively. The data obtained in three experiments, wherein the original pH of the medium varied slightly, are superimposed on the charts. The

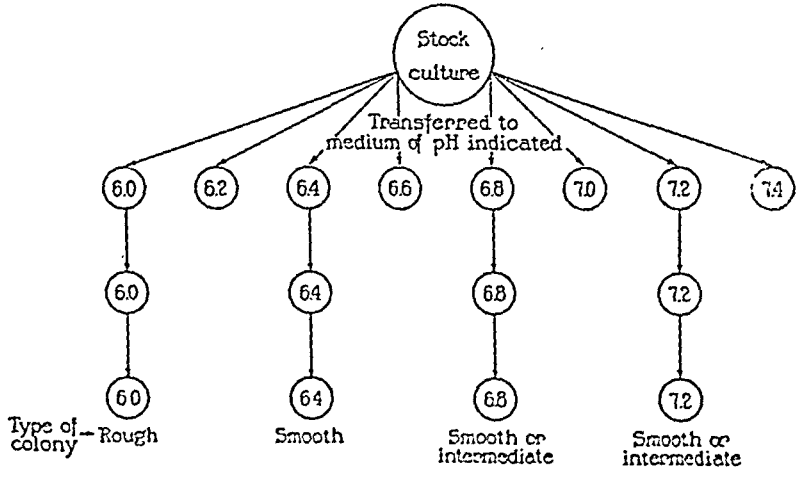

CHART 3

portions of the two curves represented as broken lines are so indicated because basal calculations were necessary in these regions and no absolute data were available. From these charts it may be seen that the addition of a definite quantity of either buffer, as indicated on the graphs, to medium of known original pH would give a final pH within 0.1 pH or less of a desired value. For absolute precision, titration curves for medium of various original pH values, with many additions of buffer, would be necessary. The methods employed, however, are wholly adequate for this type of work.

After the pH of each flask of medium had been adjusted, 5 to 7 cc. portions of the latter were placed in culture tubes of 20 to 25 cc. capacity, and the slants were inspissated in the usual manner. Sterility of the medium was determined by incubating at 37°C. for 2 to 4 days.

Since we desired only to determine the influence on dissociation of that environ-

mental change occasioned by variations in pH of the medium, no attempt was made to select colonies of different topography for transplanting. A quantity of organisms was merely removed from the parent culture with a platinum spatula and triturated just above the fluid level of saline in a small flask or tube; the container was shaken to suspend the organisms. Suspensions were prepared sufficiently dilute that colony topography could be ascertained. In order to avoid the possibility of eliminating variants which have a marked tendency to clump, the bacterial suspensions were not filtered. Transfers of each bacterial suspension were made in triplicate or duplicate by seeding each tube with 0.3 to 0.4 cc. of the suspension. In the first generation of cultures on medium of adjusted pH, all cultures of a given strain of organism were seeded from a single suspension of the stock culture. In subsequent generations transfers were also made from suspensions but always from medium of one pH to medium of the same pH value. Chart 3 illustrates this point and indicates certain general features of the colony topography observed at each pH.

Chart 3 shows the means by which subcultures of each strain of tubercle bacilli were derived in this study. The notations at the lower border indicate the pH values at which various colony variants appeared in greatest number.

After seeding, the cotton plug in each tube was covered with paraffin (m.p. 56–58°C.) and the tubes were incubated at 37°C. Each culture was twice examined with a binocular dissecting microscope during the 3rd and 4th or 5th weeks of incubation, and again after about 6 months. At each examination records were made pertaining to the amount of growth, the morphological variants present on each slant, and the approximate per cent of the whole which each comprised. The results to be discussed are those obtained in a study of three generations each, of twenty-five strains of organisms. In the first generation the medium was adjusted to eight different pH values so that there were approximately 600 cultures. Four values for pH of the medium were employed in the second and third generations, so that the whole number of cultures involved in this study was but slightly under 1,200. Contaminated cultures were discarded and not considered. They comprised less than 2 per cent of the total number of cultures.

RESULTS

Marked variation in colony topography of organisms grown at various pH values occurred in eighteen strains in the first generation. The remaining seven strains (all human type) showed similar changes in the second generation. Since the variations differed with the type of organisms, these will be discussed separately.

Avian Type Tubercle Bacilli.—At pH 6.0 and pH 6.2 a moderate number of typical rough colonies occurred in each generation, but these were most numerous in the first generation; and in each of the three generations the avian R variant (Kahn, see Table I) showed greater numbers of irregular, tortuous, granular

colonies than did the avian S or TS strains. However, even at pH 6.0 and 6.2 each strain of avian organisms showed many rounded, glistening colonies which were called smooth. Through the pH range 6.4 to 7.0 all the colonies of each of the avian strains were moist, shiny, rounded, glistening, and non-pigmented. The avian S and TS colonies were morphologically indistinguishable, although the former is avirulent and the latter highly virulent. At pH 7.2 (and pH 7.4, first generation) there was a tendency for the colonies to be flatter, but they were still regular in contour, glistening, and devoid of granular characteristics. Growth was slightly less vigorous above pH 7.0. After 6 months the smooth topography of the avian S and TS strains was maintained with little or no change; the avian R variant, however, showed secondary growth of "wormy," granular colonies, and some of the smooth colonies showed a narrow, spreading, veil-like border.

The avian strains at the various pH values showed differences in the ease with which they could be suspended in saline. The colonies grown at pH 6.4 and 6.8 were more easily suspended than those grown at pH 6.0 or pH 7.2. At pH 6.0 the organisms showed a moderate tendency to clump, while at pH 7.2 the growth was sometimes difficult to suspend, not because of a tendency to flocculate but because of a slimy stickiness which made dispersion difficult.

It is clear that smooth variants of avian tubercle bacilli, whether derived from virulent or avirulent strains, were produced over a broad range of pH; the rough variants appeared only on the more acid medium and even then not to the exclusion of smooth forms.

Bovine Type Tubercle Bacilli.—Each of the twelve bovine strains showed variations in topography dependent upon the pH of the medium in the first generation. At pH 6.0 and pH 6.2 the colonies were predominantly granular; some of them were characteristic, dry, tortuous, irregular, coarsely granular forms, rising sharply from the medium but adhering to its surface. These were designated rough. Other colonies, present in greater number, were more finely granular and had a tendency to spread over the surface of the medium; with prolonged incubation these became tortuous, "wormy," and matted together, and sometimes developed pale yellow pigmentation (especially bovine Strain 37). Small numbers of opaque, rounded colonies were present in the first weeks of incubation but became stippled or knobby in appearance later and often developed a granular veil. The latter type of colonies constituted about 11 per cent of the growth, whereas the granular variants constituted about 89 per cent. In the second and third generations the morphologic variants were similar but more moist in appearance until aged.

At pH 6.4 through pH 6.8 the granular variants of bovine strains showed a sharp decline in numbers, while the smooth, rounded, glistening colonies were most numerous. The latter constituted 23 to 90 per cent of all colonies in this pH range. They were usually quite moist and often semitransparent and somewhat jelly-like

in appearance. At these pH values the growths were composed almost exclusively of finely granular, spreading, veil-like colonies (intermediate) and glistening, rounded, non-granular forms (smooth). Smooth non-granular colonies of bovine tubercle bacilli grown at pH 6.4 and pH 6.8 are illustrated in Figs. 1 and 2 respectively. Fig. 2 may be compared with Figs. 5 and 9 of human tubercle bacilli grown at the same pH (6.8). These photographs illustrate the broader range for smooth colonies among bovine type organisms. With aging, the veil-like colonies became wrinkled but less "wormy" and matted than similar colonies grown on more acid medium; the smooth colonies, upon aging, often lost lustre and sometimes showed secondary growth of a veil-like border, or of superimposed colonies, but in general their topography was quite well preserved.

At the more alkaline pH values the bovine type bacilli produced almost exclusively a third type of colony which we have designated intermediate. These colonies were low, flat, spreading, semitransparent forms, usually with rounded margins and often with elevated centers. In some instances they were glistening and homogeneous; in others finely granular, but eventually most of them became finely granular. Truly rough colonies occurred but rarely and then only in the first generation. A moderate number of smooth appearing colonies were present in the early stages of growth but these eventually acquired the topography of the intermediate forms described above.

The bovine type bacilli then produced three variants instead of two, as in the case of avian organisms. The more granular forms occurred most abundantly on the more acid medium, the smooth forms in the mid-range of pH, and the third variant, designated intermediate, was produced almost to the exclusion of other forms on neutral or alkaline medium. Organisms grown at pH 6.0 were least readily suspensible, those grown at pH 7.2 somewhat more readily, while the organisms grown at pH 6.4 and pH 6.8 were in general much more readily suspensible.

Human Type Strains.—In the first generation of cultures grown on medium of various pH values, seven human strains showed only moderate differences in colony morphology, whereas three strains (including H-37) showed sharp variations depending on the pH of the medium. In the second and third generations all strains showed similar variations. The morphologic variations induced among the human type strains by altering the pH of the medium were in many respects like those noted among bovine type strains. Again three principal colony forms were observed. And again the more granular colonies were more numerous on the most acid medium, while the intermediate colonies were most numerous on the more alkaline medium. The apparent optimum range of pH for the development of smooth, glossy colonies was narrower than among bovine types however.

At pH 6.0 (and pH 6.2 in the first generation) granular, irregularly shaped colonies were more numerous than at other pH values. The number of these rough forms increased in successive generations from 2 to 8 and then 18 per cent of the whole number of colonies. At this pH, however, the predominating colonies were finely granular and somewhat veil-like, becoming wrinkled, tortuous, and "wormy" with aging and drying. The first generation of human strains showed no smooth colonies at pH 6.0, but in the second and third generations there were moderate numbers of glistening colonies, some of which became knobby or mulberry-like with age. Granular variants of human strains grown at pH 6.0 are illustrated in Figs. 3 and 7.

At pH 6.4 the smooth colonies were more numerous than at the other pH values, constituting from 25 to 47 per cent of the whole number of colonies. A very few rough colonies (1 to 5 per cent) were present, the remainder being of the variety designated intermediate. The smooth colonies of human strains were quite similar to the smooth variants of bovine organisms, but in general they were slightly less moist and slightly less regular in contour. Smooth variants of two human strains grown at pH 6.4 are illustrated in Figs. 4 and 8; they may be compared with smooth variants of a bovine strain in Figs. 1 and 2, and contrasted with granular variants of the same human strains grown on more acid medium (Figs. 3 and 7). The first generation of human strains grown at pH 6.6 showed fewer smooth colonies than the same cultures grown at pH 6.4, but more than were present at other pH values, indicating that the optimum pH may be slightly above pH 6.4. These smooth variants showed the same changes with age as the corresponding bovine variants: loss of lustre and growth of secondary colonies which were not smooth.

Through the pH range 6.8 to 7.2 (and 7.4 in the first generation) the predominating colonies closely resembled the bovine intermediate variants. They showed a marked tendency to spread over the surface of the medium. They were fairly regular in contour, often showed elevated centers, and were finely granular in appearance. In general these intermediate colonies of human tubercle bacilli were less glossy than the corresponding bovine variants. With aging and drying they frequently became wrinkled and tortuous. Moderate numbers of smooth colonies occurred at pH 6.8 and fewer at pH 7.2, especially in the early weeks of incubation, but these were far less numerous than at pH 6.4. Coarsely granular or rough variants were seldom seen either at pH 6.8 or 7.2, except after the culture was considerably aged.

The human type strains therefore produced variants similar to those of bovine tubercle bacilli. In the case of the bovine organisms, however, the optimum range of pH for smooth colonies on Corper's medium was pH 6.4 to 6.8, whereas among human strains large numbers of smooth colonies occurred only at pH 6.4. The pH range for intermediate variants of human tubercle bacilli was therefore

in appearance. At these pH values the growths were composed almost exclusively of finely granular, spreading, veil-like colonies (intermediate) and glistening, rounded, non-granular forms (smooth). Smooth non-granular colonies of bovine tubercle bacilli grown at pH 6.4 and pH 6.8 are illustrated in Figs. 1 and 2 respectively. Fig. 2 may be compared with Figs. 5 and 9 of human tubercle bacilli grown at the same pH (6.8). These photographs illustrate the broader range for smooth colonies among bovine type organisms. With aging, the veil-like colonies became wrinkled but less "wormy" and matted than similar colonies grown on more acid medium; the smooth colonies, upon aging, often lost lustre and sometimes showed secondary growth of a veil-like border, or of superimposed colonies, but in general their topography was quite well preserved.

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The bovine type bacilli then produced three variants instead of two, as in the case of avian organisms. The more granular forms occurred most abundantly on the more acid medium, the smooth forms in the mid-range of pH, and the third variant, designated intermediate, was produced almost to the exclusion of other forms on neutral or alkaline medium. Organisms grown at pH 6.0 were least readily suspensible, those grown at pH 7.2 somewhat more readily, while the organisms grown at pH 6.4 and pH 6.8 were in general much more readily suspensible.

Human Type Strains.—In the first generation of cultures grown on medium of various pH values, seven human strains showed only moderate differences in colony morphology, whereas three strains (including H-37) showed sharp variations depending on the pH of the medium. In the second and third generations all strains showed similar variations. The morphologic variations induced among the human type strains by altering the pH of the medium were in many respects like those noted among bovine type strains. Again three principal colony forms were observed. And again the more granular colonies were more numerous on the most acid medium, while the intermediate colonies were most numerous on the more alkaline medium. The apparent optimum range of pH for the development of smooth, glossy colonies was narrower than among bovine types however.

At pH 6.0 (and pH 6.2 in the first generation) granular, irregularly shaped colonies were more numerous than at other pH values. The number of these rough forms increased in successive generations from 2 to 8 and then 18 per cent of the whole number of colonies. At this pH, however, the predominating colonies were finely granular and somewhat veil-like, becoming wrinkled, tortuous, and "wormy" with aging and drying. The first generation of human strains showed no smooth colonies at pH 6.0, but in the second and third generations there were moderate numbers of glistening colonies, some of which became knobby or mulberry-like with age. Granular variants of human strains grown at pH 6.0 are illustrated in Figs. 3 and 7.

At pH 6.4 the smooth colonies were more numerous than at the other pH values, constituting from 25 to 47 per cent of the whole number of colonies. A very few rough colonies (1 to 5 per cent) were present, the remainder being of the variety designated intermediate. The smooth colonies of human strains were quite similar to the smooth variants of bovine organisms, but in general they were slightly less moist and slightly less regular in contour. Smooth variants of two human strains grown at pH 6.4 are illustrated in Figs. 4 and 8; they may be compared with smooth variants of a bovine strain in Figs. 1 and 2, and contrasted with granular variants of the same human strains grown on more acid medium (Figs. 3 and 7). The first generation of human strains grown at pH 6.6 showed fewer smooth colonies than the same cultures grown at pH 6.4, but more than were present at other pH values, indicating that the optimum pH may be slightly above pH 6.4. These smooth variants showed the same changes with age as the corresponding bovine variants: loss of lustre and growth of secondary colonies which were not smooth.

Through the pH range 6.8 to 7.2 (and 7.4 in the first generation) the predominating colonies closely resembled the bovine intermediate variants. They showed a marked tendency to spread over the surface of the medium. They were fairly regular in contour, often showed elevated centers, and were finely granular in appearance. In general these intermediate colonies of human tubercle bacilli were less glossy than the corresponding bovine variants. With aging and drying they frequently became wrinkled and tortuous. Moderate numbers of smooth colonies occurred at pH 6.8 and fewer at pH 7.2, especially in the early weeks of incubation, but these were far less numerous than at pH 6.4. Coarsely granular or rough variants were seldom seen either at pH 6.8 or 7.2, except after the culture was considerably aged.

The human type strains therefore produced variants similar to those of bovine tubercle bacilli. In the case of the bovine organisms, however, the optimum range of pH for smooth colonies on Corper's medium was pH 6.4 to 6.8, whereas among human strains large numbers of smooth colonies occurred only at pH 6.4. The pH range for intermediate variants of human tubercle bacilli was therefore

slightly more broad than in the case of the bovine organisms. Intermediate colonies of two human strains grown at pH 6.8 are shown in Figs. 5 and 9. Note the difference between these and colonies of bovine type bacilli grown at the same pH, Fig. 2. The intermediate colonies of the same human strains grown at pH 7.2 (Figs. 6 and 10) closely resemble those grown at pH 6.8.

DISCUSSION

Although it seems improbable that any one factor yet ascertained is wholly responsible for dissociation of tubercle bacilli, the results reported indicate the profound influence which a single factor, namely, pH of the culture medium, may have in determining the colony form of tubercle bacilli. That this is not the sole determining factor can be seen from the fact that pH control does not in any instance (except with avian bacilli) eliminate every variant except one. With the mammalian strains used, there were at least two variants present at each pH, although the results are striking enough when cultures grown at one pH value are compared with cultures of the same strain grown at a different pH value.[3] It must be stressed, however, that with prolonged incubation which allows for secondary growth, the influence of pH control becomes less evident. It is essential therefore in pursuing such a study that observations be made at least by the time cultures have been incubated for 1 month. Moreover, it is equally essential that some means of study be employed whereby colonies may be magnified four to ten diameters.

We have employed tubes as containers rather than plates because of greater assurance against contaminations and against drying of the medium; the latter is seemingly an important factor. For photographing cultures, however, tube containers are less satisfactory. In

[3] The recent work of Deskowitz and Shapiro (11) may bear upon, indeed, may offer an explanation for some of the facts under discussion. In a study of *Salmonella aertrycke* these workers found that S variants gave rise only to S variants, whereas R variants gave rise to both R and S daughter colonies. Under constant conditions a parent R colony gave rise to constant numbers of R and S daughter colonies. When environmental conditions were changed, the number of S colonies (derived from an R parent) was greatly increased, owing not to a deviation from the dissociation constant, but to the circumstance that the altered environment permitted a more rapid growth of the S cells.

order to overcome this difficulty, the entire slant may be removed from the tube with a sterile platinum spatula and placed in a sterile dish which is then covered with plate glass through which exposures are made.

The avian tubercle bacilli are seemingly less sensitive to alterations of pH than the mammalian strains. However, the avian and mammalian strains behave similarly in one respect, namely, the production of granular colonies on more acid medium. Although we have failed to observe flat, spreading, finely granular colonies of avian bacilli, these undoubtedly occur, as they have been observed by Petroff (10). It may be that the range of pH employed in our studies was not sufficiently broad (on the alkaline side) for the development of this variant. Smooth variants of avian strains of either high or low virulence are produced on Corper's medium over a wide range of pH.

Among mammalian strains, three principal variants occur. These we have designated rough, smooth, and intermediate. Rough forms of both bovine and human type bacilli occur in greatest numbers on the more acid medium used (pH 6.0). Smooth colony forms of human strains occur in greatest number at pH 6.4 and pH 6.6, whereas bovine smooth forms also occur in large numbers at pH 6.8. In both instances neutral or alkaline medium favors the intermediate variants.

The virulence of nineteen of the strains included in this study has been previously studied and reported (6). In the study of pathogenic properties, only the undissociated stock cultures of each strain were used. It was shown that among bovine type organisms the strains varied markedly in virulence from very low to very high, and that cultures of either high or low virulence, when recovered from inoculated animals, showed similar variations in colony topography; quantitatively, however, the more smooth colonies could be recovered from animals inoculated with the more virulent strains. Now in the present study variations in topography of colonies, occasioned by alterations of the pH of the culture medium, show that strains of low and of high virulence produce similar morphologic variants. If it could be established that none of the variants derived from avirulent strains possess enhanced pathogenic properties, then it could be concluded that dissociation is a general bacteriological phenomenon which occurs among non-pathogenic Mycobacteria, as well as among

pathogens. Such a conclusion would in no wise detract from a further statement that the major pathogenic properties of a virulent strain are associated with some one variant. Such studies have been made; the results, which confirm these views, will be discussed in a subsequent report.

It may be well to mention, however, that tests of virulence of some of the variants obtained in the present study have been made, and that they vary strikingly in pathogenic properties. The bovine rough variant derived from a highly virulent strain is only moderately pathogenic; the smooth variant is at least as virulent, if not more so, than the parent strain; and the variant designated intermediate on account of its colony form is truly intermediate in pathogenesis. None of the variants of an avirulent bovine strain showed enhanced pathogenic properties. Variants of the Burroughs human strain showed moderate differences in pathogenic properties. The pH 6.4 culture produced the most extensive lesions. The details of these experiments are mentioned here only to facilitate discussion.

These facts indicate that a delicate mechanism operates to determine both colony form and virulence of tubercle bacilli. If the factors of that mechanism are controlled so that maximum numbers of smooth colonies are produced, then greatest virulence is retained. The bacilli, when grown on more acid medium, produce fewer smooth and greater numbers of rough colonies and suffer attenuation. Sensitivity to alkaline medium is less, so that still another and but moderately attenuated colony variant is produced. It seems likely that this latter is the explanation for the fact that the pH range most suitable for the development of intermediate colonies does not occur between those values which are more suitable for R or S variants.

It cannot be assumed that the colonial variation due to pH would be identical were some culture medium other than glycerol-egg yolk medium used. It is possible that with some other culture medium the pH values optimum for certain colonial variants might be quite different from those observed in the present study.

The difference in optimum pH for development of smooth colonies which occurs between human and bovine tubercle bacilli might possibly be used as an additional means of typing new strains. In order to make the method effective, however, considerable experience in the

study of variants would be necessary; and one would need to study several cultures made on medium of each pH value.

It is interesting and perhaps noteworthy that the pH best suited to development of smooth colonies of mammalian tubercle bacilli is practically identical with that of the vacuoles in the cells which phagocytize the bacilli in the animal body (the vacuoles of monocytes react to neutral red showing a pH of about 6.5). This may indicate that at least one host factor is not unfavorable to the invading bacterium.

SUMMARY

The colony topography of tubercle bacilli is significantly affected by altering the pH of the culture medium on which the organisms are grown. Under the conditions of these experiments, avian tubercle bacilli produce two variants, rough and smooth. The former are most numerous on the most acid medium used (pH 6.0); the smooth colonies are obtained over a broad range of pH.

Three colonial variants of bovine and human tubercle bacilli are described. Both mammalian types produce greater numbers of rough colonies at pH 6.0. The bovine type strains produce greatest numbers of smooth colonies in the pH range 6.4 to 6.8, and intermediate colonies on alkaline medium. The human type strains produce greatest numbers of smooth colonies at pH 6.4 and large numbers of intermediate colonies at pH 6.8 and pH 7.2.

Included among the avian and bovine strains studied are organisms of widely varying pathogenic properties. Virulent and attenuated strains of a given type produce similar colonial variants under similar environmental conditions.

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EXPLANATION OF PLATES

PLATE 6

FIG. 1. Smooth colonies of a bovine strain of tubercle bacilli, grown on Corper's medium at pH 6.4. Age of culture, 29 days. ×8.

FIG. 2. Smooth colonies of a bovine strain of tubercle bacilli grown on Corper's medium, pH 6.8. Age of culture, 29 days. ×8.

FIG. 3. Rough colonies of the Burroughs strain, human tubercle bacilli, grown on Corper's medium at pH 6.0. Age of culture, 36 days. ×8.

FIG. 4. Smooth colonies of the same strain as Fig. 3, grown on the same medium at pH 6.4. Age of culture, 36 days. ×8.

FIG. 5. Intermediate colonies, same strain as Figs. 3 and 4, grown at pH 6.8. Compare with Fig. 2, showing bovine tubercle bacilli cultivated under identical conditions. Age of culture, 36 days. ×8.

FIG. 6. Intermediate colonies, same strain as Figs. 3, 4, and 5, grown at pH 7.2. Age of culture, 36 days. ×8.

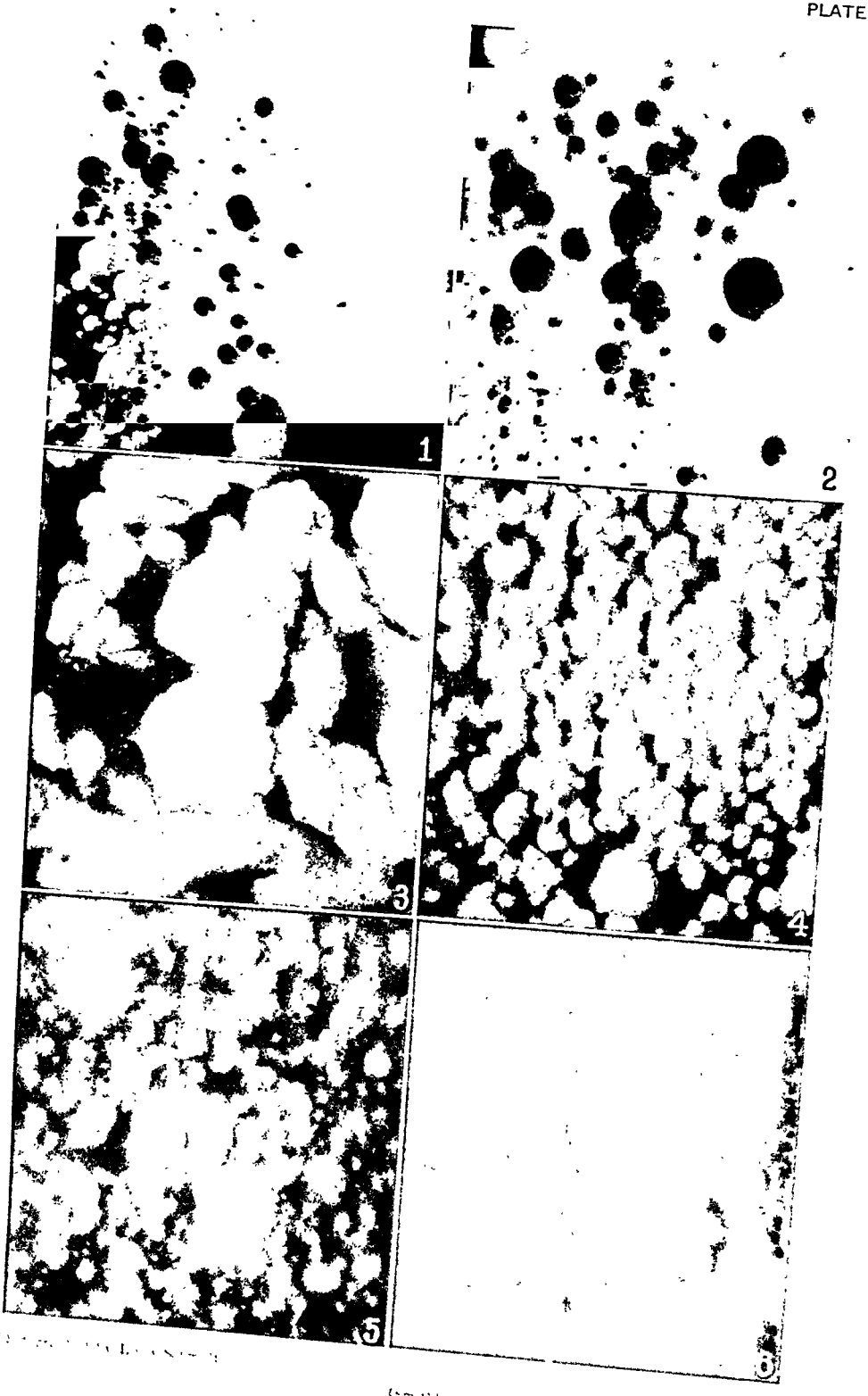
PLATE 7

FIG. 7. Rough colonies of another human strain, Jamaica, grown on Corper's medium at pH 6.0. Age of culture, 105 days. ×8.5.

FIG. 8. Smooth colonies of the Jamaica strain, grown at pH 6.4. Age of culture, 105 days. ×8.5.

FIG. 9. Intermediate colonies of the Jamaica strain, grown at pH 6.8, also 105 days old. ×8.5.

FIG. 10. Intermediate colonies of the Jamaica strain, grown at pH 7.2. Age, 105 days. ×8.5.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EXPERIMENTS ON ACTIVE IMMUNIZATION AGAINST EXPERIMENTAL POLIOMYELITIS

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The results of many efforts to induce resistance in *Macacus rhesus* monkeys against experimental poliomyelitis by means of inoculations of virus in one form or another have been summarized by Stewart and Rhoads (1929) (1) and in the volume published by the Milbank International Committee (1932) (2). What may be derived from these experiments, beginning with the first, undertaken 25 years ago by Flexner and Lewis (3), is that "it is impossible to protect monkeys by the use of killed virus and second, that a definite though inconstant resistance to poliomyelitis can be brought about by the intradermal and subcutaneous introduction of the living virus" (1). The fact also emerges from the numerous trials hitherto reported that resistance is acquired by monkeys when a sufficient amount of active virus is given intra- or subcutaneously in one massive dose (3, 4) or in smaller amounts repeated over a considerable period of time (3–6). Even then protection is not afforded to some animals and the degree of immunity induced varies in others, while now and again a treated monkey succumbs to the disease as a result of the inoculations (1, 5–8).

Two noteworthy series of articles have recently appeared, in one of which was described the immunity obtained through the use of virus completely inactivated by 0.1 per cent formalin (Brodie, 9) and in the other the protection conferred with active but ricinoleated virus (Kolmer and his associates, 10). While the principles underlying both methods had already been employed (2), the recent investigators report results which lead them to believe that immunity can be safely induced with their materials.

Since on the basis of Brodie's and Kolmer's work widespread inoculations of children against poliomyelitis have been undertaken recently,

it was deemed desirable to restudy this problem, following as closely as possible the methods of these investigators. The intention was to determine whether any advance has been made in the experimental immunization of monkeys over that which has been accomplished in the past 25 years, and whether any procedure has been disclosed that might be practical for immunization of man.

For the purpose of comparison we also studied another form of chemically treated virus as immunizing agent, namely, that precipitated by tannin, which will be described first.

Methods

Virus.—The animals selected as source of poliomyelitis virus were extensively paralyzed and moribund as a result of the experimental disease. They were killed by ether inhalation and the spinal cord removed under aseptic conditions. The identification of the particular virus used in the preparation of each immunizing agent was ascertained by (*a*) animal inoculation with production of specific clinical signs and pathological changes, and by (*b*) neutralization with specific homologous strain antiserum. The M.V. and Philadelphia strains (11) of virus were employed.

Method of Testing for Acquired Active Immunity.—Monkeys were tested for induced resistance by the inoculation of homologous strains intracerebrally and intranasally.

The intracerebral test dose[1] consisted of 0.2 cc. of 5 per cent fresh poliomyelitis cord suspension which was filtered through a Berkefeld N filter. Kolmer (10), working with the M.V. strain, states that one infective unit was contained in 0.05 cc. of an unfiltered suspension in some instances and in 0.2 cc. in others. However this may be, the high cost of monkeys makes it impractical to titrate each individual virus sample; hence the test dose for induced resistance should be one that experience has shown to be unequivocally effective. The dosage as given in the following experiments has been consistently employed in this laboratory for many years with satisfactory results. Normal monkeys receiving it react with the experimental disease within, as a rule, 5 to 11 days; only exceptionally does an animal resist. All the controls of the following series of tests developed the characteristic infection.

The intranasal test for induced immunity consisted of the instillation into each nasal cavity of 1 cc. of 10 per cent glycerolated cord suspension, and after 1 or 2 days' interval the treatment was repeated. The reaction was measured not only by clinical signs but also by cell counts of the spinal fluid withdrawn daily through cisternal puncture. The method is essentially that of Flexner (12) and his associates and suffices satisfactorily to determine the state of immunity in a treated

[1] All such inoculations were made with the aid of full ether anesthesia.

animal. It may be said that the amount as given is not too drastic since in a collateral series of twenty-four monkeys, twenty-one developed poliomyelitis; the three unaffected ones could not be considered immune, only uninfluenced by the treatment, since one of them—the only one retested—was later shown to be susceptible to a similar intranasal instillation of virus. Hence the test dose as practised is in the range of minimal infective dosage. It is of interest that all controls so treated which were employed in the experiments to be reported were successfully infected.

Test for Antiviral Bodies in Serum.—0.8 cc. of undiluted serum is mixed with 0.2 cc. of 5 per cent filtered fresh cord virus, kept at 37°C. for 2 hours and in the cold for 16 to 18 hours, and then injected into the brain of monkeys. For control, the serum is replaced by physiological saline solution. Here again neutralization tests are carried out with the homologous strain of virus. The test can be regarded as a practical one even though the precise titration of antibody content of a serum is not ascertained.

Tannin-Precipitated Virus as Immunizing Agent

In a correlated study on the virus of equine encephalomyelitis, it was found that vegetable-derived tannin (tannic acid) precipitated the proteins of the tissue containing the virus and the latter precipitated with the proteins remained infective although somewhat reduced in potency (13). The virus could not be designated as "attenuated" but merely as present in lesser amounts in the flocculated substance. Under these conditions the infective agent retained its activity for several weeks. As the following will show, similar results were obtained with tannin precipitates of active poliomyelitis tissues.

Preparation of Immunizing Agent.—2.5 gm. of poliomyelitis cord were thoroughly ground with sand and suspended during the grinding in 50 cc. of distilled water. The suspension was spun in an angle centrifuge for 15 minutes at 2,000 R.P.M. The supernatant fluid only was retained and was decanted into a 100 cc. centrifuge flask and 5 cc. of 2 per cent aqueous solution of Mallinckrodt's tannic acid were added. The mixture was energetically shaken and then stored overnight in the cold. After about 18 hours the material was again shaken and centrifuged for 20 minutes at 3,000 R.P.M. The supernatant fluid was discarded and the precipitate washed, with stirring, in 50 cc. of Tyrode's solution. After similar centrifugation, the sediment was collected and resuspended in 50 cc. of hormone broth, pH = 7.6. This suspension was stored in the cold and used as immunizing agent from 3 to 14 days after its preparation.

The tannin-precipitated virus was injected subcutaneously in the amounts to be mentioned and in several instances produced locally small, indurated masses which regressed after 1 or 2 weeks.

Results of Preventive Inoculations.—It will be seen from Table I that of eight monkeys injected subcutaneously with the 5 per cent virus suspension (M. V. and Philadelphia strains), two died of non-poliomyelitis affections and of the remaining six, two became moribund after an attack of poliomyelitis following the first subcutaneous dose of 2 cc. In the one instance in which the antigen was Philadelphia virus, 1 cc. inoculated intracranially induced poliomyelitis in a control monkey. When the immunizing agent was reduced in content of virus to 1 per cent of cord by weight and only a total of 4 cc. of it was given subcutaneously to each of four monkeys and 1 cc. intracerebrally to a fifth, none of the five so treated developed disease.

The data in Table I clearly show that material containing active virus can by itself give rise to fatal infection after a single subcutaneous injection. It is significant, however, that of two monkeys receiving three such doses of the same virus sample, one failed to be protected against a subsequent intracerebral test inoculation but the second resisted the intranasal test instillation. Results based on the reactions of only two animals are inconclusive but they serve to bring out one of the difficulties met with in attempting to immunize animals with active virus preparations.

The power of the various tannin-precipitated virus preparations to build up resistance was not great, for it is noted that of three monkeys receiving the Philadelphia virus and which were given the intracerebral test inoculation, all developed poliomyelitis. Of the treated animals injected with the 5 per cent antigen and tested intranasally for immunity, two succumbed and one monkey was found to be resistant to this and a repeated test. Of those receiving the 1 per cent material, both were resistant to the first intranasal test dose but were susceptible to a second test.

Hence only one of the five animals receiving the immunizing agent was found resistant to the intranasal test and that one resisted both of two tests. Even so, one cannot regard this monkey as immune, for, as Flexner (14) shows, monkeys can be refractory to several successive courses of instillations yet respond to a final one of the same virus. In addition, these results confirm Flexner's finding that when virus is placed in contact with the nasal mucosa, pleocytosis may occur, but the increase in the number of cells in the spinal fluid may not be

associated with symptoms of infection or with the development of immunity.

The capacity of tannin-precipitated virus to produce serum antiviral bodies is varied. Of the series injected with 5 per cent virus antigen, the pooled serum of two monkeys and individual sera of two others neutralized virus by the method described; of the animals given the 1 per cent antigen, the pooled serum of two neutralized and that of two others failed to do so. To be noted is that in five instances treated monkeys yielded neutralizing serum but were found, 51, 71, and 301 days after the last immunizing dose was given, to be susceptible in average degree to intracranial or intranasal contact with virus. This is not unusual; it has recurred in the experiments soon to be described with formalin and ricinoleate. Moreover, Stewart and Rhoads (1), Schultz and Gebhardt (15), and recently Aycock[2] and others have reported the lack of correlation existing between serum antiviral bodies and immunity as tested by the cerebral or nasal routes. In other words, the presence in the monkey of serum antiviral bodies, as produced by artificial immunization and determined by the described method, is no definite indicator of the state of active resistance of the animal to the test doses used.

To summarize the results of preventive treatment with tannin-precipitated poliomyelitis virus, it would appear that this product has failed as a satisfactory immunizing agent and that it is restricted by the same uncertainty which living virus as such manifests as a preventive when injected under similar conditions. Too much of the material can induce infection; too little, inconstant and unreliable immunity.

Active Ricinoleate-Treated Virus as Immunizing Agent

Kolmer (10), basing his experiments on those of McKinley and Larson (16), employed 1 per cent sodium ricinoleate to attenuate but not inactivate the poliomyelitis virus in 4 per cent cord suspensions prepared from 1 month old glycerolated tissue. The ricinoleated material was kept in the cold for 1 month before use and then in one series of experiments 0.1 cc. of the agent per kilo body weight was injected subcutaneously five times at 5 day intervals into seven monkeys, and similar dosages were given intracutaneously to three additional animals. They

[2] Personal communication.

showed no symptoms, and 1 month after the last treatment, when subjected to an intracerebral inoculation of 0.2 cc. of 5 per cent virus suspension, one developed poliomyelitis and the others were unaffected. The survivors were again injected intracerebrally with virus up to 17 months later and all but one survived a third cerebral test for resistance.

In repeating the experiment with sodium ricinoleate-treated virus, we used the same virus (M.V. strain) which Kolmer employed and the sodium ricinoleate was sent us through the kindness of the same manufacturers.[3] The methods were those of Kolmer except as regards the intracerebral test dosage: Kolmer employs as a test dose for induced immunity unfiltered and we, filtered suspensions. In addition, we employed nasal instillation, as described, for this purpose, a procedure which he omitted.

Results of Preventive Inoculations with Ricinoleate-Treated Virus.—Reference to Table II shows that six monkeys received the Kolmer vaccine. Of two tested intracerebrally for immunity, both failed to resist and of four instilled intranasally, two developed the disease on the first instillation and a third on a repeated test. Thus only one of the six animals resisted the tests for acquired resistance.

Table II reveals that the pooled serum of two treated animals and the individual sera of the remaining four neutralized virus in each instance. Here again, as occurred with tannin-precipitated virus, the antiviral bodies, as determined by the method given, were present but despite this fact the animals succumbed to the tests for active immunity.

When these experiments were well advanced, a paper was published by Schultz and Gebhardt (15), which stated: "The serums of another series of animals 'immunized' earlier with *living* virus (Kolmer vaccine) neutralized 30 M. I. D. doses of virus per cubic centimeter, but when these animals were subjected to intranasal instillation with active virus, they all developed typical poliomyelitis." We can thus confirm the findings of these investigators.

Formolized Virus as Immunizing Agent

In preparing materials, the methods of Brodie (9) were followed. 0.2 per cent formalin was added to 20 per cent active cord suspensions in equal volumes so that in the end 0.1 per cent formalin was in contact with 10 per cent virus suspension. This was kept at 37°C. for 16 hours, since at the time when this work was

[3] William S. Merrell and Co., Cincinnati.

TABLE II

Ricinoleate-Treated Virus as Immunizing Agent, Employing 4 Per Cent Glycerolated Poliomyelitis Cord Suspension + 1 Per Cent Sodium Ricinoleate, Subcutaneously

Monkey No.	Immunization		Tests for active immunity					Tests for passive immunity	
	Amount* M. V. virus antigen 5 doses, 5 d. intervals	Result	Route	Dose given about 30 d. after last I. D.	Result	When retested with M.V. strain nasally (2 doses)	Result	Serum procured after last I.D.	Result
								days	
16	0.3 cc. (total 1.5 cc.)	N.S.	I.C.	M.V. virus	P. 6 d.			20	Pooled serum neutralized; (control, P. 6 d.)
17	0.27 cc. (total 1.35 cc.)	"	"	" "	P. 7 d.			20	
18 (control)	None	—	"	" "	P. 4 d.				
19	0.3 cc. (total 1.5 cc.)	N.S.	I.N.	M.V. virus 2 doses	N.S.	67 d. after last test	P. 11 d. (4 controls P. 7, 9, 10, 11 d.)	20	Serum neutralized; (control, P. 6 d.)
20	0.35 cc. (total 1.75 cc.)	"	"	" "	P. 8 d. sp.fl. 29–644 cells			20	Serum neutralized; (control, P. 6 d.)
21	" "	"	"	" "	P. 8 d. sp.fl. 45–730 cells			17	Serum neutralized; (3 controls, 2 P. 6 d. and 1, 22 d.)
22	0.34 cc. (total 1.7 cc.)	"	"	" "	N.S.	67 d. after last test	N.S. (4 controls same as Monkey 19)	17	Serum neutralized; (2 controls, P. 14 d. and 3 d.)
23 (control)	None	—	"	" "	P. 7 d. sp.fl. 330 cells				
24 (control)	1 cc. injected I.C.	P. 9 d.							

Abbreviations same as in Table I.

*0.1 cc. ricinoleate-treated virus per kilo.

TABLE III

Formolized Virus as Immunizing Agent,
Employing 0.1 Per Cent Formalin Fresh 10 Per Cent Virus (Cord) Suspension, Kept for 16 Hours at 37°C., Intradermally

Monkey No.	Immunization		Tests for active immunity					Tests for passive immunity	
	Amount given (2 x 5 cc. at 13 d. intervals) Strain	Result	Route	Dose	Result	When re-tested intracerebrally	Result	Serum procured after last I.D.	Result
								days	
25	Phila.	N.S.*	I.N.	Phila. virus 2 doses 30 d. after last I.D.	P. 8 d. sp. fl. 24–834 cells			21	Pooled serum. Monkey febrile 7 to 11th d. T = 104.8°F.† Excited. Recovery (partial neutralization?); (2 controls, both P. 8 d.)
26	"	"	"	" "	P. 9 d. sp.fl. 17–224 cells			21	
27 (control)	None	—	"	Phila. virus 2 doses	P. 8 d. sp.fl. 22–420 cells				
28	Phila.	N.S.	I.C.	Phila. virus 36 d. after last I.D.	N.S.	57 d. after 1st test dose	N.S.	21	Pooled serum. Monkey febrile 7th to 13th d. T = 105°F., slight ptosis. Recovery (partial neutralization?); (2 controls, P. 8 d.)
29	"	"	"	" "	P. 6 d.			21	
30 (control)	None	—	"	Phila. virus	P. 7 d.				
31 (control)	"	—	"	" "	P. 7 d.				
32 (control)	2 cc. of formolized Phila. virus I.C.	N.S.							

33	M.V.	"	I.N.	M.V. virus 2 doses 30 d. after last I.D.	P. 8 d. sp.fl. 27–460 cells			20	Pooled serum. No neutralization; (control, P. 6 d.)
34	"	"	"	" "	P. 10 d. sp.fl. 33–340 cells			20	
35 (control)	None	—	"	M.V. virus 2 doses	P. 8 d. sp.fl. 25–514 cells				
36	M.V.	N.S.	I.C.	M. V. virus 50 d. after last I.D.	P. 8 d.			20	Pooled serum neutralized; (control, P. 10 d.)
37	"	"	"	" "	P. 5 d.			20	
38 (control)	None	—	"	M.V. virus	P. 8 d.				
39 (control)	"	—	"	" "	P. 7 d.				
40 (control)	2 cc. of formolized M.V. virus I.C.	N.S.							

Abbreviations same as in Table I.

*The monkeys injected with formolized virus showed local skin necrosis with ultimate healing within about 2 weeks.

†T = highest temperature reading during febrile course.

done, Brodie stated that 12 to 16 hours of such contact served to inactivate poliomyelitis virus, and that the 16 hour material was employed by him as immunizing agent.

With respect to dosage for immunization of monkeys, it was first stated by Brodie (17) that one dose of 5 cc. yielded as good results as two doses of 5 cc.; this was later (18) changed so that it was then declared that two injections were more efficacious than a single intradermal one of 5 cc. In the following experiments, however, two doses of 5 cc. each were used throughout.

The intracerebral test for induced resistance as employed by Brodie was made with amounts on the borderline of infectivity, designated as "minimal completely paralyzing doses." In Table III, the intracerebral test was the same as given in the foregoing series of experiments with tannin and sodium ricinoleate, so that a proper comparison could be made of the different methods of immunization. This consisted of 0.2 cc. of filtered 5 per cent fresh cord suspensions. No mention is made by Brodie of determining immunity by means of intranasal instillation of virus; this we have carried out along with the intracerebral test.

Results of Preventive Inoculation with Formolized Virus.—As will be seen in Table III, of eight monkeys injected with formolized virus, only one resisted, and that one was found refractory to two successive intracranial test inoculations. It is common experience among workers in this field to meet with an occasional monkey refractory to poliomyelitis virus, so that it is uncertain whether the animal in question was immunized by the formolized material or not.

Of four sets of pooled serum, as indicated in Table III, one showed neutralization, another, none, and a third and fourth so called incomplete neutralization, due perhaps to low antibody content. The lack of correlation between serum antibodies with active protection has already been commented upon.

It is therefore plain that this method offers, under the experimental conditions employed, an ineffective immunizing material against poliomyelitis in monkeys.

The experience of Schultz and Gebhardt (15) employing the same agent is as follows:

> They injected fifteen monkeys: three subcutaneously, four intramuscularly, and four intradermally, giving 0.1 cc. per kilo of 0.1 per cent formolized 10 per cent virus, and four intravenously with ten times this amount, five times at weekly intervals. 24 days after the last immunizing dose the animals received three M.I.D. of virus. "All developed the disease in about the same length of time, and with about as extensive paralysis as the controls, despite the fact that their serums seem

to have acquired slight, but definite virucidal properties." In additional experiments, Schultz and Gebhardt (15) injected the immunizing agent repeatedly in the brain of four monkeys and instilled it repeatedly in the nasal cavities of four others. All eight were proved susceptible to later inoculation with virus, in the brain in the first series and in the nose in the second.

The results we have obtained are corroborative of those of Schultz and Gebhardt, although the latter investigators employed a lesser amount of vaccine, and lead to the conclusion that formolized virus is not an effective preventive against poliomyelitis in the monkey. Other earlier observers (Abramson and Gerber, 19; Römer, 20; and Jungeblut and Engle, 21) also did not succeed in inducing immunity by means of formolized poliomyelitis virus.

DISCUSSION

The object of this study was the investigation of the problem of active immunization of *Macacus rhesus* monkeys by means of chemically treated poliomyelitis virus. The materials employed were tannin-precipitated virus and virus treated with sodium ricinoleate and with formalin. The latter two substances are those with which the vaccines of Kolmer and Brodie respectively are prepared and the tannin material introduced by us was employed for comparative observations.

The virus of poliomyelitis treated with tannin or sodium ricinoleate retains its activity so that intracerebral inoculation of monkeys with the preparations induces characteristic experimental poliomyelitis. Indeed, Kolmer (10) records that 0.2 cc. of his vaccine kept for 5 months when so inoculated induced the disease within 12 days. Further, the tannin-precipitated virus itself brought on infection in two animals after a single subcutaneous injection of 2 cc. It is therefore plain that the chemical treatment in both instances did not act to attenuate the virus.

The results of the experiments can be summarized by stating that if the immunizing agent contains a sufficient amount of virus, the danger arises of infecting an animal with the material itself. Under the experimental conditions employed, these preparations, although active virus was present in them, failed to immunize the inoculated animals regularly. Serum antiviral bodies were, however, produced

by means of the described methods but it was shown that animals in which these antibodies were present did not resist the ordinary tests for active immunity.

From what is here reported, it is apparent that there is no advantage to be derived from the use of the tannin-precipitated, or ricinoleated virus as immunizing agents over unchanged active virus, as employed in the past in this laboratory (Flexner and Lewis, 3; Flexner and Amoss, 22; Stewart and Rhoads, 1; and Rhoads, 23) and elsewhere (Aycock and Kagan, 5, and others).

A study of the recorded experiments of the past 25 years on immunization of monkeys reveals that active poliomyelitis virus itself is not a potent antigen, as are some other viruses; uniform protection is rarely brought about through its use. A greater degree of success in protecting animals can, however, be achieved when large doses over long periods of time are employed—which fact might lead one to suppose that the difficulty with poliomyelitis virus as immunizing agent may be related simply to the amount of antigenic substance present. Some viruses, such as those of equine encephalomyelitis (24) and yellow fever[4] among several others, can be diluted to 10^{-8} and still be infective for the most susceptible host, whereas poliomyelitis virus can be diluted to only a fraction of this amount to reach the limit of infectivity in the monkey. It is still unknown why the antigenic capacity of this virus is relatively less than that of several others. Finally, if amounts of virus sufficing to produce disease in some monkeys but not in others confer no immunity on the unaffected ones, it is to be expected that a lesser amount would be even less effective.

We now come to an estimation of formolized virus. In this instance, the evidence of earlier observers (2), later of Brodie (9, 17), of Schultz and Gebhardt (15), and ourselves points to the inactivation of the virus by the chemical. It is still an open question whether any form of inactive poliomyelitis virus retains the property of immunizing animals (2). An analysis of the results of the present investigation shows that active immunization with formolized virus by the Brodie method does not build up resistance in monkeys to the usual intracerebral or intranasal tests for induced immunity. The amount of antiviral bodies produced in the serum by this vaccine is slight and, as already indi-

[4] Theiler, M., personal communication.

cated, the treated monkeys failed, notwithstanding the presence of antibodies, to resist the tests for active immunity.

There are, therefore, discrepancies in the conclusions of Brodie (9) and ourselves. These may perhaps be ascribed to the fact that Brodie employs borderline dosages in his tests. With such small doses, it is possible that certain monkeys may not receive what for them is an infective dose of virus. At this point we wish to emphasize the fact that the intranasal test dose for immunity employed here was within the range of a minimal infective dose, as we have pointed out before; nevertheless, animals receiving formolized virus (or tannin-precipitated or ricinoleated virus) and among them even those which possessed serum antiviral bodies were found to be susceptible to this test.[5]

There remain for consideration the factors derived from animal experimentation which either Kolmer or Brodie maintains as a basis for the claims that a safe and successful immunizing agent has been made available for use in man.

The first factor which Kolmer (10) stresses as the essential one is the non-infectivity of his preparation. Kolmer admits that the degree of attenuation by sodium ricinoleate is slight or of minor importance but safety is acquired through the use of remote monkey passage virus that has apparently lost its infectivity for man. There is no experimental evidence for this assumption (25, 26).

The second is that ricinoleated and formolized vaccines engender in monkeys serum antiviral bodies and that the same mechanism might apply in man. It has been shown by Schultz and Gebhardt (15) and

[5] As this article goes to press, Brodie states (*J. Am. Med. Assn.*, 1935, 105, 1089) that virus suspensions should be "just inactivated, for overtreatment or prolonged treatment with solutions of formaldehyde reduced the antigenicity of the vaccine," and therefore recommends the use of virus inactivated for 8 to 12 hours instead of 16. The distinction between "just inactivated" and "overinactivation" is not clear. In view of the still more recent modification (18) of 5 to 6 hours' contact with 0.1 per cent formalin at 37°C., it is apparent that this vaccine contains active virus as shown by Brodie in experiments in which 6 hours' treatment fails to inactivate the virus. The amount of active material may be small since, as Brodie points out, monkeys develop the disease only after repeated inoculation of 6 hour treated suspensions. It is known, however, that such small amounts of active virus do not induce protection in monkeys; still the possibility of infection during the period of immunization with an agent that contains active virus is ever present.

THE RELATION OF LEUKOSIS TO SARCOMA OF CHICKENS*

II. Mixed Osteochondrosarcoma and Lymphomatosis (Strain 12)

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Plates 8 to 10

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Some transmissible neoplasms in chickens result from the growth of a single type of cell and others from the growth of two or more types of cells. In the present work the transmissible diseases produced in successive passages by injection of tissues or tissue extracts deriving from a chicken having either leukosis or sarcoma, or both, will be designated strains, and we shall distinguish between simple and complex strains, as just characterized. The sarcomata of Rous (1) and the erythroleukosis of Jármai (2) are examples of simple strains, while our Strain 13 (3) is a complex strain resulting from the growth of endothelium and of primitive erythroblasts. Similar complex strains have been described by Oberling and Guérin (4) and by Rothe Meyer and Engelbreth-Holm (5).

The discovery that avian sarcoma (1) and leukosis (6) are produced by viruses affords ready explanations for the occurrence of complex strains by admixture. It may be argued that one virus may stimulate to multiplication several types of cells, but it is as equally conceivable that the complex strains are produced by two or more viruses. This question is still unsettled and it will be dealt with here.

The finding of a new complex strain (No. 12), here described, gave opportunity to undertake such a study. Osteochondrosarcoma occurred in a chicken that was inoculated intravenously with blood of a chicken having lymphomatosis of Strain 2. Strain 2 itself is complex (7). Although the majority of the chickens inoculated with it develop lymphomatosis, in many birds there is evidence of growth of

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basophile erythroblasts, myelocytes, and endothelial cells; Strain 2 does not produce bone tumors. Among the passages of Strain 12 there occurred osteochondrosarcoma or the type of neoplasms produced by Strain 2, mainly lymphomatosis or a combination of these neoplasms. Our first series of experiments was based on the assumption that a bone tumor virus had contaminated the virus of leukosis of Strain 2 and we attempted to separate these two hypothetical agents. It has been possible to reisolate leukosis of Strain 2 by procedures here described; but attempts to isolate a virus that produces bone tumor unassociated with leukosis have thus far been unsuccessful.

Viruses causing osteochondrosarcoma in chickens have already been described by Rous, Murphy, and Tytler (8, 9) and Muto (10). According to Tytler (9) the tumor first mentioned (Chicken Tumor VII) appeared to have arisen from the periosteum, and the neoplastic tissue consisted of spindle-shaped or multipolar cells of fibroblastic type. The tumor now to be described is a neoplasm of osteoblasts and its virus does not stimulate common connective tissue cells, while the virus of the Chicken Tumor VII of Rous, Murphy, and Tytler produced neoplasms when injected into the voluntary muscles, by affecting the connective tissue cells there present.

Origin of Strain 12

The origin of Strain 12 in the fourth passage of Strain 2 is indicated in Text-fig. 1 of the paper that describes transmission experiments with Strain 2 (7).

No. 3293, a young Barred Rock chicken weighing approximately 900 gm., was injected Dec. 15, 1932, in the wing vein with 0.05 cc. blood of a chicken with lymphatic leukemia of Strain 2. Apr. 21, 1933, a firm fusiform tumor approximately 9 x 4 x 4 cm. was found at the site of injection encircling the right humerus. A part of the bony tumor was removed from the anesthetized bird and was transplanted into 3 chickens. 6 days later, chicken 3293 was moribund; it was killed and another transplantation of the tumor attempted (Text-fig. 1 and Table I).

At autopsy the cortex of the humerus was thickened, ill defined, and its lumen, normally air-containing, was infiltrated with tumor tissue (Fig. 1). The growth extended by direct continuity into the cavity of the chest. The lungs were thickly and uniformly studded with partly confluent gray-white or hyaline gray tumor nodules varying in size from 0.1 to 1 cm. and replacing about one-half of the lung

tissue. The blood-forming organs (bone marrow, spleen, and liver) showed no changes suggestive of leukosis. Microscopic appearances of the wing tumor are shown in Figs. 5 and 6. The soft parts of the tumor were composed of large round or polygonal cells which were either detached or arranged like epithelial cells. Their nucleus was large vesicular or oval, poor in chromatin, containing one or, occasionally, two large nucleoli (Fig. 8). The firm parts of the tumor showed on microscopic examination formation of cartilage and to a lesser extent bone (Fig. 5). There was abundant cartilage formation in the metastatic tumors in the lungs.

Transmission Experiments

Earlier in the course of these studies the transplantation of tumors was made by cutting the tumors into small fragments in the presence of Locke or Tyrode solutions and injecting a suspension of tumor particles intramuscularly. Later the technic was modified by bringing the tumor tissue in proximity to normal bones. The inoculations in the wing were made by injecting tumor tissue about the periosteum of the humerus. On withdrawing the needle, minute amounts of tumor tissue were introduced into the muscle and subcutaneous tissue. The inoculations in the breast were made by piercing the sternal keel and injecting small particles of the tumor into the muscle tissue and into the sternal keel.

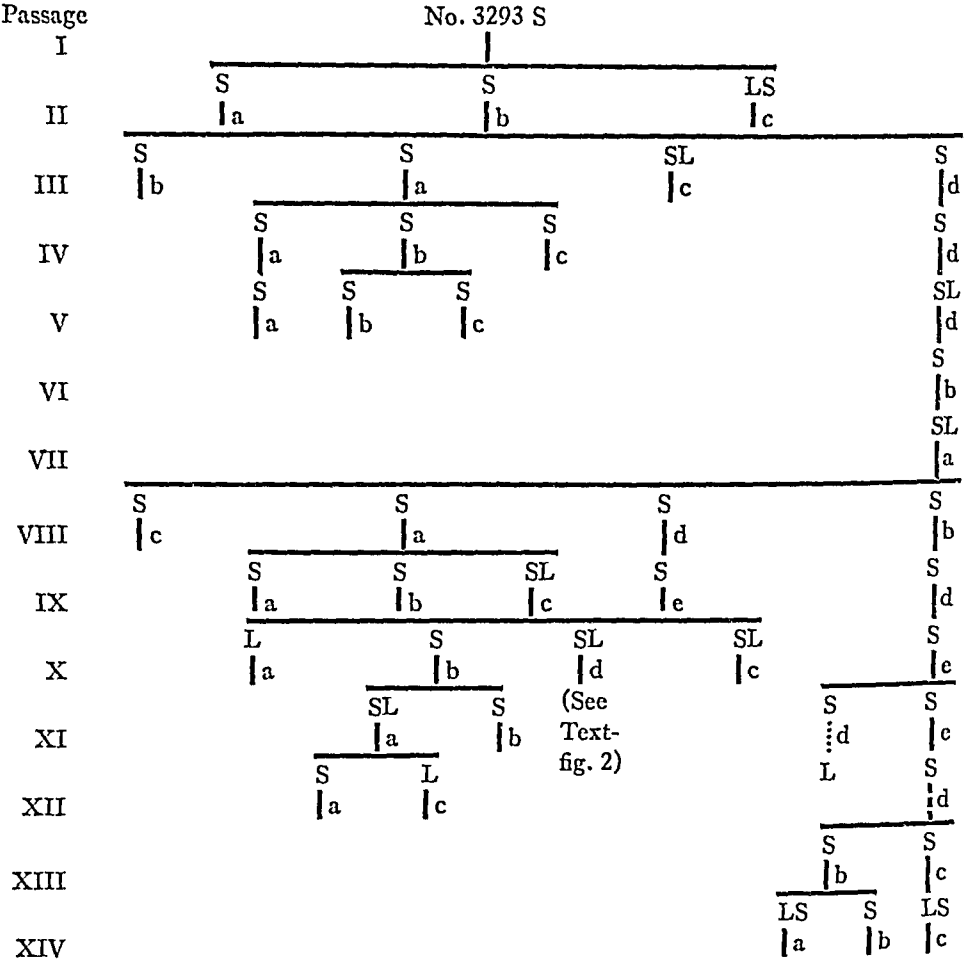
Two of 8 birds receiving intramuscular injections of tumor tissue of chicken 3293 developed sarcoma; in 2 others the small tumors that developed at the site of injection regressed; four remained healthy (Text-fig. 1 and Table I). Numerous subpassages were made, but several attempted transfers were unsuccessful, and our major problem was to keep this strain alive. This necessitated frequent intramuscular transplantations of tumor tissue, a procedure that we hoped might result in the elimination of the leukosis virus. At the same time experiments were undertaken to demonstrate whether the osteochondrosarcoma is caused by a virus, and if so, to preserve it.

Text-fig. 1 gives a survey of the first 40 passages, made between May, 1933, and April, 1935. It shows only the chickens from which subpassages were made and the results of individual passages are given in Table I. The striking feature of these inoculations is that among the passages made from chickens with apparently pure bone sarcoma there occurred again bone sarcoma and leukosis.

Chickens that died of intercurrent diseases within 1 month after inoculation and showed no evidence of leukosis or tumors were discounted from the total number of injections; those that died after 1 month were given among the unsuc-

cessful injections. In a few chickens the tumors produced by inoculation regressed; they were counted arbitrarily among the successful injections.

The majority of chickens used were Barred Rocks. The few White Leghorn and hybrid chickens tested proved equally susceptible. The age of the chickens



TEXT-FIG. 1. Passages of Strain 12.

S = sarcoma; L = leukosis of the types produced by Strain 2, mainly lymphomatosis; SL = sarcoma and leukosis; — = inoculation unsuccessful. Route of injection: | = intramuscular (i.m.); ⋮ = intravenous (i.v.); ¦ = intramuscular and intravenous.

varied from a few weeks to a few years. In contrast to most strains of leukosis and sarcoma, young chickens were not more susceptible to Strain 12 than older ones, and the high incidence of fatal intercurrent diseases among young chickens made it desirable to use mature chickens in experimental studies of this disease.

TABLE I

Results of Inoculations with Cell-Containing Material of Strain 12

No. of passage	Material injected	Route of injection	No. of chickens injected	No. of successful injections			No. of unsuccessful injections
				Bone sarcoma	Bone sarcoma and leukosis	Leukosis	
I	Tumor particles	i.m.	8	2		2	4
II a	" "	"	4				4
II b	" "	"	9	5	2		2
II c	" "	"	1			1	
III a	" "	"	4		1	1	2
III b	" "	"	1	1			1
III c	" "	"	5	1			1
III d	" "	"	4				4
IV a	" "	"	5				3
IV b	" "	"	3				5
IV c	" "	"	2				3
IV d	" "	"	3	3			2
V a	" "	"	2				
V b	" "	"	3			1	2
V c	" "	"	3				2
V d	" "	"	3	1			3
VI a	" "	"	4		1		2
VII a	" "	"	14	4	2	2	6
VIII a	" "	"	11	5	4		2
VIII b	" "	"	4	3			1
VIII c	" "	"	2				2
VIII d	" "	"	2	1	1		2
IX b	" "	"	3	1			
IX c	" "	"	4		3		2
IX d	" "	"	3	1	2		1
IX e	" "	"	2		2		
X a	" "	"	4		1		
X b	" "	"	5	3	2	3	
X c	Blood	"	3				
X e	Tumor particles	"	3	3		3	
XI a	Blood and tumor cell suspension	i.v.	4				
XI b	Tumor particles	i.m.	4	2		2	
XI d	" "	"	1		1	1	
XI e	Blood and tumor cell suspension	i.v.	5	1	1	1	
XII a	Tumor particles	i.m.	4	2	1	3	
XII c	" "	"	2	1			
XII d	" "	"	2		1	1	
XIII b	and blood Tumor cell suspension	i.v.	3	2	1	1	
XIII c	Tumor particles	i.m	6	1	1	2	
Total			169	45 (27%)	30 (17%)	17 (10%)	77 (46%)

Table I shows that 54 per cent of the transmissions with living cells were successful. Of the injected chickens 27 per cent had sarcoma, 10 per cent leukosis, and 17 per cent had both. Five successive subpassages, made from chickens with sarcoma and apparently free from leukosis, shown in the last vertical column of Text-fig. 1, did not change the ability of this strain to produce leukosis. If Strain 12 consists of two viruses, one that produces bone tumor and another that produces leukosis, the leukosis virus is obviously present in all chickens with seemingly pure osteochondrosarcoma, but we have no knowledge concerning the conditions that maintain the existence of the leukosis virus in the chickens with sarcoma.

The numerous unsuccessful inoculations induced us to try to increase the susceptibility of the experimental birds by irradiation with X-rays, but without success.

The following is a summary of two experiments (passages IIIb and IVd).

Unirradiated chicks.....K 33 sarcoma, D 35 sarcoma, D 52 sarcoma, K 56 negative, D 194 negative.

Irradiated with 350 r.....D 18 negative, D 125 negative.

Irradiated with 500 r.....K 12 sarcoma, K 16 sarcoma and leukosis, K 18 negative, D 45 sarcoma and leukosis, K 58 negative.

Similar results which were obtained with different viruses that produce leukosis or tumor led us to abandon further attempts to increase the susceptibility of chickens by X-rays. When younger chickens are used, the loss due to X-ray injuries is considerable and irradiation of large numbers of mature chickens is expensive.

An attempt to separate the hypothetical sarcoma agent from the leukosis agent was made by injecting diminishing amounts of tumor tissue into healthy chickens, but without success.

The bird from which passage VIII a was made showed no blood involvement of leukosis. Nevertheless, minute amounts of its bony tumor (0.2 cc. of a dilution of 1:10,000) produced leukemic lymphomatosis with no tumor at the site of injection. The tumor tissue diluted 1:100 produced both sarcoma and leukosis.

Tumor cell suspension	Result of injection
Undiluted	D 56 sarcoma and leukosis, D 76 negative, D 131 sarcoma
1:100	K 69 sarcoma and leukosis, K 87 sarcoma, K 107 negative
1:10,000	K 79 leukosis, D 105 neurolymphomatosis, K 112 leukosis

The few cases of neurolymphomatosis occurring among the passages of this strain may be regarded as spontaneous (11).

Similar results obtained with a strain of sarcoma (endothelioma) associated with leukosis (Strain 13 (3)) indicate that it is unlikely that experiments of this kind will yield the sarcoma virus free from the leukosis virus.

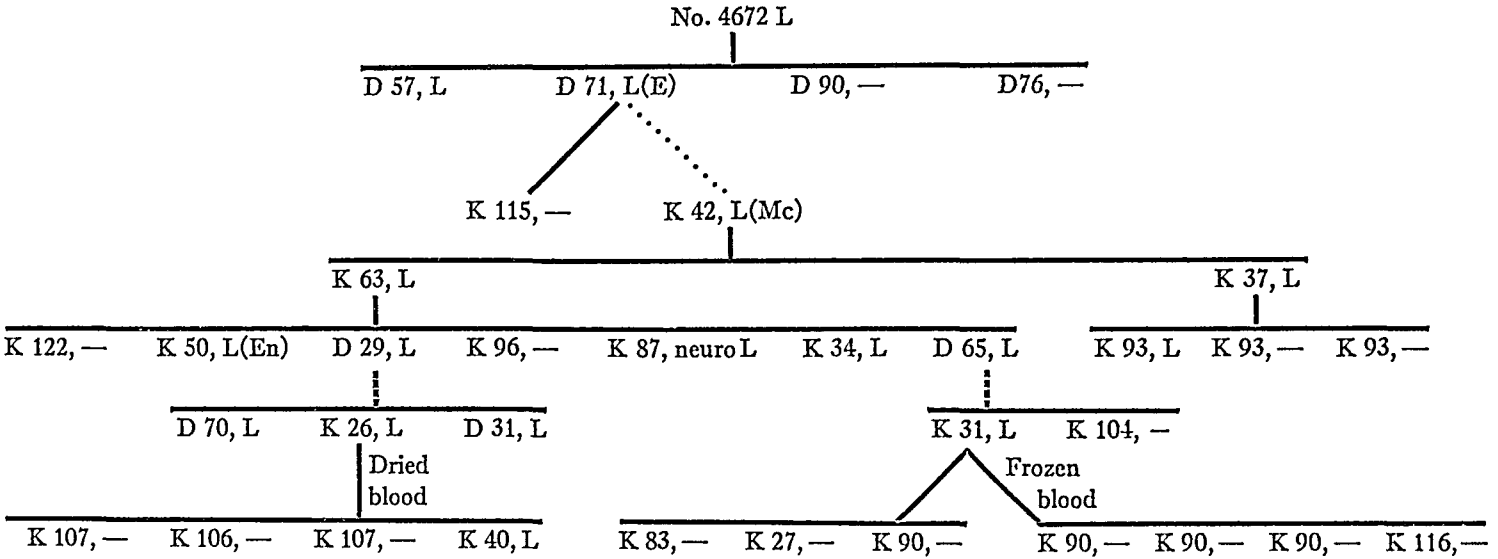
Separation of the Leukosis Agent.—The experiment described suggested that even in tumor tissue the concentration of the leukosis agent is greater than the concentration of the sarcoma agent, and attempts were made, therefore, to separate the leukosis agent from the sarcoma agent.

Clotted blood of a chicken that had lymphomatosis was injected into the breast of 4 chickens, piercing the sternal keel in a similar manner to that used for the transmission of osteochondrosarcoma. 2 of the 4 chickens injected developed leukosis free from sarcoma. The results of further subpassages are shown in Text-fig. 2. Osteochondrosarcoma did not occur in this series involving 34 chickens, of which 15 developed lymphomatosis of the type produced by Strain 2, and 1 neurolymphomatosis.

In the experiments shown in Table II, fresh blood was used for inoculation, unless stated otherwise. Injections made with the blood of another leukotic chicken (No. 5425) that was inoculated with osteochondrosarcoma tissue and developed lymphomatosis unassociated with bone tumor, yielded results similar to those shown in Text-fig. 2. 2 of 4 chickens injected in the breast died of lymphomatosis and 2 of intercurrent disease.

These observations support the assumption that Strain 12 consists of a mixture of sarcoma and leukosis agents; the latter is of greater virulence, is present in the blood in more active form or in a greater concentration, and can for this reason be separated from the virus of osteochondrosarcoma.

Since most of these passages were made with cell-containing material, it is probable that the disease was the result of the multiplication of immature blood cells, and that in many chickens the virus did not come in contact with susceptible bone cells. For this reason two experiments were made: (*a*) Desiccated blood of a chicken (No. 5183, Table II) with presumably pure leukosis was injected through the sternal keel, but it produced lymphomatosis without bone tumor.



TEXT-FIG. 2. Subpassages made from a chicken with lymphomatosis occurring among the passages of Strain 12.
L = lymphomatosis, E = erythroleukosis, Mc = myelocytomatosis, En = endothelioma, — = unsuccessful injection. D = died; K = killed. The figures after these letters show the number of days after inoculation. Most chickens were killed when moribund, a few because of intercurrent infection.

TABLE II

Inoculation with Desiccated and Frozen Tumor of Strain 12

No. of donor	Virus free from live cells			Fresh tumor (control)	
	Age of material	No. of chickens injected	No. of successful injections	No. of chickens injected	No. of successful injections
	days				
Desiccated tumor					
Experiment 1					
3226	15	3	0	3	0
3622	26	3	0	4	0
3646	62	3	0	4	1 (SL), 1 (L)
Experiment 2					
3920	19	6	1 (S)	6	1 (S), 3 (SL), 2 (L)
3852	75	6	0	—	—
3823	133	6	0	3	2 (S)
3646	154	6	1 (S)	4	1 (SL), 1 (L)
3708	56	6	0	2	0
3226	107	6	0	3	0
Experiment 3					
3226	366	6	0	3	0
3920	278	6	1 (S)	6	1 (S), 3 (SL), 2 (L)
4380	140	6	0	2	2 (S)
4009	227	6	0	2	2 (S)
4728	1	6	2 (S)	4	3 (S)
4730	21	6	0	1	0
Frozen tumor					
3920		5	2 (S)	5	1 (S)
3920		2	0	4	2 (S), 2 (L)
4009		2	0	2	1 (S), 1 (SL)
4229		4	1 (S), 1 (SL)	4	2 (S), 2 (SL)
5172		4	0	4	1 (SL)

Notes on Table II.—All injections were made in the wing and breast muscles and in the proximity of bones. Fresh and frozen tumor tissue was injected at different sites into the same chickens. Three of four dried samples were tested on each chicken, the material being injected at different sites such as wing, breast, and leg.

The capital letters in the columns headed "Successful injections" show the type of disease produced, sarcoma (S) and leukosis (L) respectively.

The fresh tumor of chicken No. 3226 was virulent; the table does not show 8 chickens that were injected with it after a previous unsuccessful inoculation with this strain, 5 of which developed tumors at the site of injection.

The virus of leukosis of Strain 2 introduced in a similar manner likewise failed to produce bone tumors (11). (*b*) The blood of a chicken (No. 5378, passage XIVa) that had bone sarcoma and leukosis was injected in the breast muscle and about the keel bone, and intravenously into 5 chickens. One, killed 43 days, another killed 116 days and a third that died 42 days after injection, had lymphatic leukemia and osteochondrosarcoma at the site of injection.

These experiments strongly support the view that leukosis of Strain 2 can be reisolated from Strain 12; they show that the virus of osteochondrosarcoma circulates in the blood of tumor-bearing chickens.

Attempts at Cell-Free Transmission.—Two procedures, desiccation and freezing and thawing (Table II), were used to demonstrate the causation of Strain 12 by an agent separable from living cells.

Tumor tissue of 12 chickens was dried in the frozen state as described in previous reports (12, 7). Successfully dried samples of viruses that produce neoplasms kept in the refrigerator usually retain their activity almost undiminished during periods of years. Table II shows that only 3 of 12 dried specimens were active. One of these (No. 3920), tested 19 and 278 days after drying, proved equally active but produced tumors in only 2 of 12 inoculated chickens.

Freezing and thawing was performed by submerging cut up tumor tissue in alcohol in a sealed test tube at −30°C. during 30 minutes. Previous experiments (12, 7) have shown that this procedure injures the viruses only slightly, but it destroys live cells. Table II shows that the agent of Strain 12 resists freezing and thawing better than desiccation in the frozen state.

Anatomical Characteristics of the Tumors

The anatomical changes in the chicken from which Strain 12 took its origin and the method of transplantation of tumor tissue and inoculations with the cell-free virus have been described above. In the chickens with tumors produced by transplantation the growth appeared about the periosteum of the humerus, remained firmly adherent to it, and extended into the muscle tissue; the humerus either remained air-containing or became filled with tumor tissue, in some cases being destroyed and lost in the new growth. In the sternum the tumors appeared as nodular prominences on either side of the sternal keel from which they could not be separated. In several chickens which died with the blood involvement of lymphomatosis and anemia, or of an intercurrent disease, a small bony projection from the sternal keel, microscopically osteochondrosarcoma or osteochondroma, was the only bone tumor found. Fig. 2 shows destruction of the sternal keel by tumor.

The growth in the breast muscle produced by similar injection with either Strain 2 (11), 13 (3), 11 or 15 (13) did not destroy the sternal keel. Separate tumors appeared on both sides of the breast muscles. Although in some chickens the bone cavities were infiltrated with tumor tissue, bone and cartilage formation occurred only in chickens that received tissues of Strain 12.

Somewhat more than one-half of the chickens remained free from blood involvement and the grafted bone tumors grew progressively, reaching a size of from 6 to 10 cm. in largest diameter, 1 to 3 months after injection.

The degree of bone formation in the inoculation tumors was variable. In rapidly growing tumors there was only slight evidence of deposition of the intercellular substance of cartilage or bone (Fig. 7). Older tumors, on the contrary, contained so much bone that the tumor could not be cut with a knife. Bone formation was most extensive in the central parts of the older tumors, whereas the peripheral parts consisted of gray or pink soft growth of osteoblasts. Slowly growing tumors almost invariably contained bone and cartilage.

Metastatic tumors formed diffuse or nodular infiltrations. Of 50 chickens with tumors at the site of inoculation, metastasis was found in 11. The scarcity of metastatic involvement may be explained in part by the fact that many chickens died of leukosis or of intercurrent diseases or were killed for experimental studies, within from 1 to 2 months after inoculation. The sites of metastasis were the liver (Figs. 3, 12), bone marrow (Fig. 10), spleen, kidney, lung, ovary, and bones. Microscopically the metastatic tumors were osteoblastomata with (Fig. 12) or without (Fig. 10) cartilage formation.

In order to determine the favorite sites of the growth of the osteoblasts of Strain 12, 5 chickens were injected intravenously with suspension of bone tumor cells filtered through absorbent cotton; the same chickens were also injected intramuscularly with unfiltered suspension. Tumors appeared in the internal organs in 4 of these chickens and in the breast of all.

In one of these chickens the tumor, microscopically osteochondrosarcoma, that grew in the breast, measured 1 cm. in largest diameter 48 days after injection and 3.5 cm. at autopsy 91 days after injection. Diffuse infiltration and 1 to 3 mm. tumor nodules formed by osteoblasts enlarged the liver to approximately twice its normal size. The spleen weighed 6 gm. Its pulp was studded with cells like osteoblasts. The heart blood and sections of organs gave no evidence of leukosis. In another of these chickens similarly injected the breast tumor measured 5 x 3 x 3 cm. 78 days after injection and showed the formation of bone and cartilage but the blood-forming organs gave no evidence of sarcoma or of leukosis. In a third chicken there were areas of cartilage formation in the breast tumor, but the growth in the internal organs (liver, kidney, bone marrow, and lung) consisted of osteoblasts with no bony or cartilagenous intercellular substance.

Somewhat less than one-half of the chickens with sarcoma at the site of inoculation showed blood and organ changes characteristic of Strain 2. The number of basophile lymphocytes in the blood varied from a few to an estimated 200,000 per c. mm. Most chickens with lymphomatosis had severe anemia with numerous erythroblasts in the blood. Myelocytes were found in a smaller number of these chickens. These leukotic blood changes, as well as the organ changes with which they were associated, have been described in previous communications (7, 11). Endothelial neoplasms similar to those produced by Strain 2 (14), also occurred among the chickens inoculated with Strain 12. Fig. 9 shows chondrosarcoma of the inoculated breast and cuboidal cells in gland-like arrangement similar to Fig. 5 of the paper that describes the morphological characteristics of the endothelial lesions produced by our Strain 2 (14). In an occasional section the endothelial growth could not be distinguished with certainty from growth of osteoblasts.

It is noteworthy that, with one exception, intramuscular inoculation of bone tumor in the manner described did not result in the formation of lymphomatous infiltration at the site of injection. Bone tumor was not produced by similar introduction of lymphomatous tumor tissue of Strain 2, but it occurred after injection with leukemic blood of chickens with osteochondrosarcoma of Strain 12.

The bone tumor of Rous, Murphy, and Tytler (8, 9) consisted of spindle-shaped cells of fibroblastic type about which cartilage was laid down, followed in many instances by the formation of true bone containing red marrow. Tumor filtrate injected into muscles produced similar growths with elaboration of cartilage and bone. The tissue changes involved were neoplastic transformation and metaplasia. Bone was obtained by continuous conversion of cartilage into calcified osteoid tissue, and osteoblasts appeared to play no part in the bone formation.

All tumors produced by cell-free virus of Strain 12 arose from preformed bones or cartilage. They were small when the experiment was terminated, the largest measuring 0.7 cm. across, and none produced metastases. None of the chickens injected with dried tumor tissue developed leukosis, although 3 of the control chickens died of this disease.

The early microscopic changes in the formation of bone tumor by

virus are shown in Fig. 4. The cortex is thicker, the Haversian canals wider, the bone corpuscles are hypertrophied and transformed into osteoblasts. Much of the old bone is resorbed, its place being taken by the proliferating osteoblasts about which new intercellular substance with the staining qualities of cartilage and bone is deposited. The newly formed bone invades the medulla of the old bone and the surrounding tissues by continuity.

The histological characteristics of the osteochondrosarcomata produced in the sternal keel of 2 chickens with blood of a tumor-bearing fowl were similar to those produced by the virus. In one of these chickens the tumor measured 2.5 cm., in the other 0.5 cm. in longest diameter, 42 days after inoculation when one chicken died and the other was killed.

The virus of Strain 12 produces neoplastic changes only when it is brought in contact with preformed bone. The proliferative cells have the morphological characteristics of osteoblasts (15); they form rapidly growing tumors with almost no intercellular substance or slowly growing bony and cartilaginous tumors, occasionally benign in appearance. Under the influence of the virus of Strain 12, cartilage and bone cells arise from or change into osteoblasts, but the common spindle-shaped mesenchymal cells do not. This finding requires further elucidation since osteoblasts are modified mesenchymal cells which, even in adult organisms, can differentiate into bone-forming tissues. Furthermore, according to Roulet (16), the osteoblasts readily dedifferentiate *in vitro* into simple mesenchymal cells. The tissue culture studies of Doljanski and of Fischer (17) suggest that osteoblasts are a distinct cell type. Sabin, Doan, and Forkner (18) found that the osteosarcomata produced by irradiation were derived from osteoblasts.

It is noteworthy that the bone tumor of Rous, Murphy, and Tytler appeared as an almost symmetrical mass on the sternal keel suggestive of a developmental anomaly. Many tumors of our Strain 12 behaved in a similar manner; they were small, 1 to 2 cm. in largest diameter, remained almost stationary in size during a period of several months and their microscopic appearance was suggestive of a benign cartilaginous growth; nevertheless they were produced by the same virus that gave rise to highly malignant neoplasms.

TABLE III

Inoculation with Tumor Tissue of Strain 12 into Chickens Resistant to a Preceding Injection with a Tumor- or Leukosis-Producing Virus

Previous injections	No. of passage	No. of chickens injected	No. of successful injections	Type of disease produced	Control (first) injections		
					No. of chickens injected	No. of successful injections	Type of disease produced
A	IV a	3	0		5	0	
Strain 12	IV b	12	5	3 (S), 2 (L)	3	0	
	V c	6	3	3 (S)	2	0	
	VII a	7	2	1 (S), 1 (SL)	14	8	4 (S), 2 (SL), 2 (L)
	VIII c	2	1	1 (S)	2	0	
	VIII d	8	2	1 (S), 1 (L)	2	2	1 (S), 1 (SL)
	Total	38	13	9 (S), 1 (SL), 3 (L)	28	10	5 (S), 3 (SL), 2 (L)
B	V c	8	1	1 (L)	2	0	
Strain 12, and 1 or 2	VII a	3	0		14	8	4 (S), 2 (SL), 2 (L)
	Total	11	1	1 (L)	16	8	4 (S), 2 (SL), 2 (L)
C	III a	9	4	3 (S), 1 (L)	4	2	1 (S), 1 (SL)
Strain 2	IV b	8	1	1 (S)	3	0	
	IV c	3	0		2	0	
	VII a	3	0		14	8	4 (S), 2 (SL), 2 (L)
	VIII c	5	4	1 (SL), 3 (L)	2	0	
	VIII d	1	1	1 (L)	2	2	1 (S), 1 (L)
	X b	5	0		5	5	3 (S), 2 (SL)
	Total	34	10	4 (S), 1 (SL), 5 (L)	32	17	9 (S), 5 (SL), 3 (L)
D	III a	3	1	1 (S)	4	2	1 (S), 1 (SL)
Strain 1	IV b	5	2	2 (S)	3	0	
	VII a	2	0		14	8	4 (S), 2 (SL), 2 (L)
	VIII a	3	0		11	9	5 (S), 4 (SL)
	Total	13	3	3 (S)	35	19	10 (S), 7 (SL), 2 (L)
E	VII d	2	0		2	2	1 (S), 1 (SL)
Strain 11							

Notes on Table III.—The injections with the different strains were made at intervals of from 2 to 4 months. The tumors that developed at the site of injections regressed in three chickens of group A (passage V c), in one of group C (passage III a), and in two chickens of group B (passage IV B), given among the successful injections. One chicken of group A (passage VIII d) and another of group D (passage VII a), given among the unsuccessful injections, died of neurolymphomatosis.

Marrow formation, a frequent finding in Chicken Tumor VII of Rous, Murphy, and Tytler, was a rare occurrence in the tumors of our Strain 12. The growth illustrated in Fig. 11 was produced by injection of tumor particles in and about the sternal keel. The chicken died 88 days later, when the tumor measured 6 x 4 x 3 cm. It was sharply circumscribed and consisted of cancellous bone, the meshes of which were filled in part by marrow showing foci of erythrocyte and granulocyte formation (Fig. 11), in part by osteoblasts forming cartilage and bone. The microscopic appearance of this growth was not suggestive of malignancy, but the large size of the tumor precludes the possibility that it was produced by the trauma of the injection, and not by the material injected. Particles of the tumor removed from the anesthetized bird 25 days before these sections were taken were injected through the sternal keel and in proximity to the right humerus of 5 chickens, 3 of which developed tumors. The pathogenesis of this growth requires further study.

Injections of Chickens Previously Resistant to Inoculations with Strain 12 or to Other Strains of Leukosis and Sarcoma

These studies have been undertaken to obtain information concerning the relation of Strain 12 to Strain 2. The results, summarized in Table III, indicate that many chickens once unsuccessfully injected with tumor tissue of Strain 12 can be reinjected with success. This is contrary to our experience with transmissible strains of leukosis and to the well known behavior of other transmissible tumors.

The conclusion can be drawn from the data of Table III that a preceding injection with Strain 12 or with leukosis of Strain 2 produces little or no immunity against Strain 12. It is possible that inoculation with the cell-free virus might yield results different from those obtained with cell-containing tissues, but the virus of Strain 12 is so weak that immunity studies with it are at present not feasible.

The percentage of successful injections was very small among the chickens that received Strain 1 (group D) or Strain 12 and 1 or 2 (group B) preceding the final injection with tumor tissue of Strain 12. However, these chickens were much older than their controls and their number is too small to permit the conclusion that the primary injections produced immunity against Strain 12.

SUMMARY AND CONCLUSIONS

Osteochondrosarcoma developed in a chicken inoculated with leukosis of Strain 2. In successive passages made by intramuscular implantation of tumor tissue deriving from this chicken there occurred (*a*) osteochondrosarcoma free from leukosis, or (*b*) leukosis free from

osteochondrosarcoma, or (*c*) osteochondrosarcoma in association with leukosis. The malignant sarcoma cells of this strain have the morphological characteristics of osteoblasts.

Implantation of tumor tissue from chickens with lesions (*a*) and (*c*) yielded both osteochondrosarcoma and leukosis. Successive passages made by intravenous and intramuscular injections with blood from chickens with leukosis yielded leukosis only. Hence it is very probable that Strain 12 consists of a mixture of an osteochondrosarcoma-producing agent and the agent of leukosis of Strain 2.

Strain 12 is readily transmitted by material containing the living malignant osteoblasts but transmission is seldom successful with virus freed from living cells. This virus produces neoplasms only when brought in contact with bone or cartilage.

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EXPLANATION OF PLATES

The sections were stained with hematoxylin and eosin-azure solutions. The magnifications are approximate. All figures illustrate osteochondrosarcoma of Strain 12.

PLATE 8

FIG. 1. Osteochondrosarcoma of the chicken from which Strain 12 originated. The bony tumor replaces most of the right humerus and invades by continuity the thoracic cavity. Both lungs are studded with tumor nodules.

FIG. 2. Osteochondrosarcoma produced by implantation of tumor cells in the sternal keel (thirteenth passage). The bony tumor projects symmetrically on either side of the sternal keel and completely replaces part of it. (The chicken died 63 days after injection.)

FIG. 3. Osteochondrosarcoma of the right humerus produced by transplantation of tumor cells. Metastatic tumors in the liver. (Second passage; chicken killed 5 months after the injection.)

PLATE 9

FIG. 4. Early osteochondrosarcoma of the humerus produced with dried tumor tissue. The chicken was killed 30 days after inoculation. × 20.

FIGS. 5 and 6. Microscopic appearance of osteochondrosarcoma of the humerus of the chicken from which Strain 12 originated. ×200.

FIG. 7. Osteoblastic proliferation with only slight deposition of cartilagenous intercellular substance produced by intramuscular implantation of tumor cells. The chicken was killed 87 days after the injection when the tumor measured 8 x 4 x 3.5 cm. There was extensive metastasis to the lung. × 180.

PLATE 10

FIG. 8. High magnification (× 900) of the malignant cells characteristic of Strain 12. The section is from the chicken with the spontaneous osteochondrosarcoma.

FIG. 9. Breast tumor showing neoplastic cartilage and gland-like structures, the latter formed by cuboidal cells, probably endothelial in origin. The chicken was injected through the sternal keel with tumor cells and died 94 days later. ×100.

FIG. 10. A small tumor nodule in the femoral marrow of the same chicken, formed by osteoblasts. The marrow contains abundant fat, is slightly congested, and shows no evidence of leukosis. × 180.

FIG. 11. Marrow formation in a growth that measured 6 x 4 x 3 cm., 88 days after injection of tumor cells in and about the sternal keel. × 100.

FIG. 12. Metastatic osteoblastic infiltration in the liver with formation of cartilage. The gross appearance of the tumor is shown in Fig. 1 and the history given in the text. × 180.

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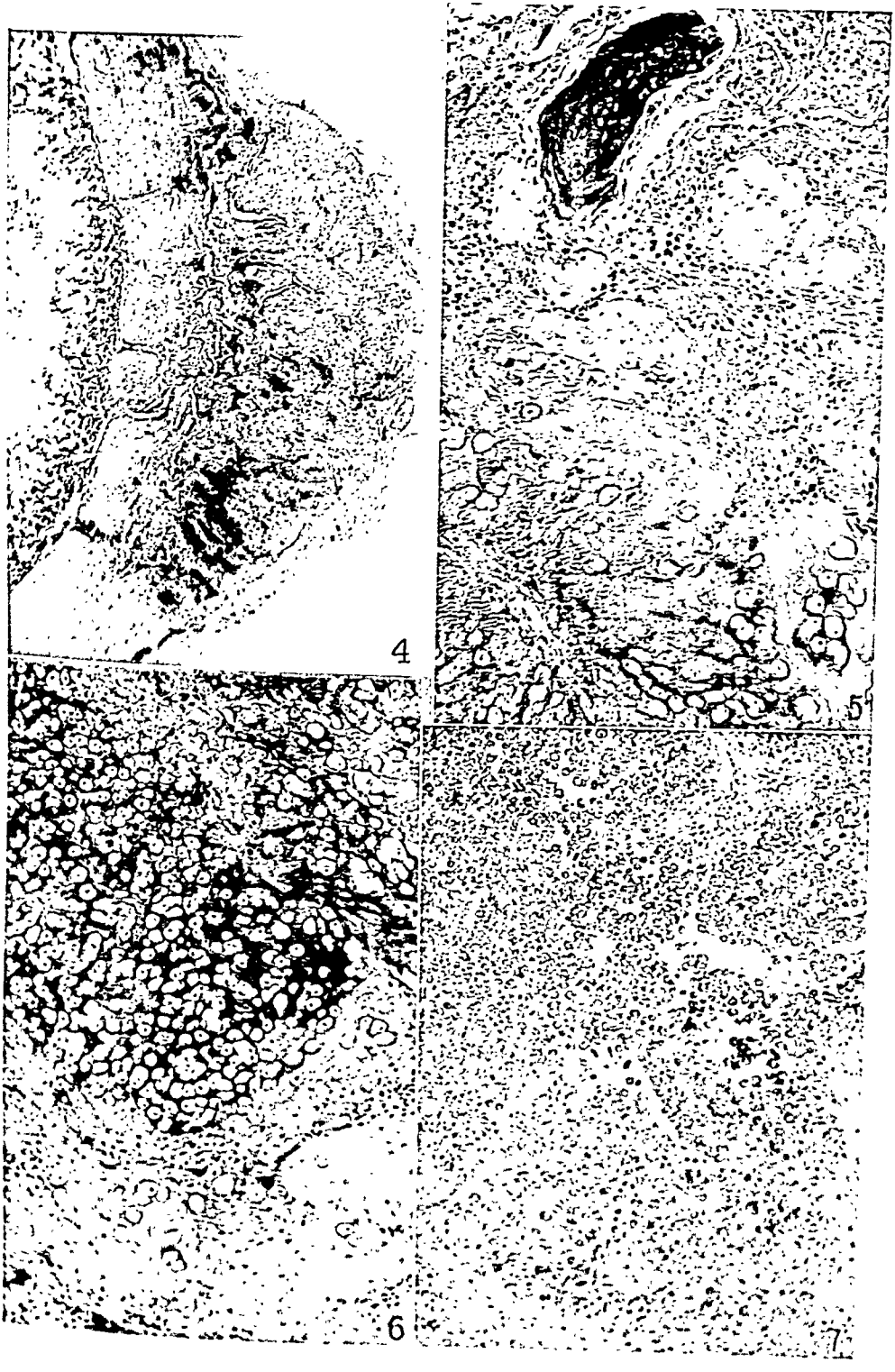
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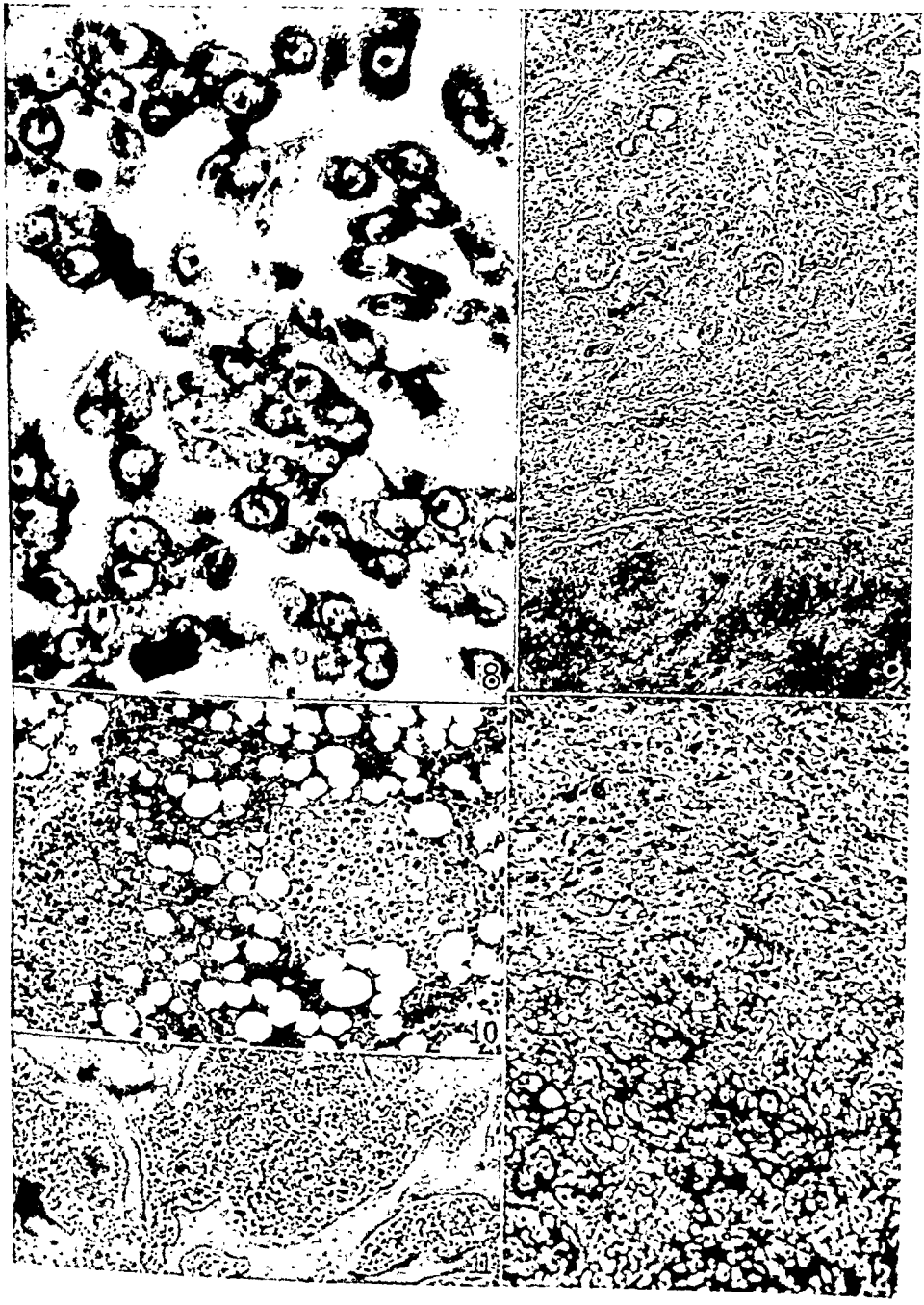

(Ford: Leukemia, or Lymphoma of chickens. II.

THE RELATION OF LEUKOSIS TO SARCOMA OF CHICKENS*

III. Sarcomata of Strains 11 and 15 and Their Relation to Leukosis

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Plates 11 to 13

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Two strains of chicken sarcoma (Nos. 11 and 15) are described in this report. Neoplasms with properties similar to that of Strain 15 have not been observed hitherto in chickens (1). Strain 11, which is similar to the spindle cell sarcoma of Rous (2) and to tumors found subsequently in several countries (1), will be described because it was used in studies on the nature of the transmissible tumor strains which produce more than one type of growth (3, 4). The following are some introductory notes about these strains, given here for comparison with Strain 12 described in the preceding paper.

No. of strain.....	12	15	11
Source	A chicken injected with leukosis of Strain 2	A chicken injected with neurolymphomatosis of Strain 6	Uninjected control chicken
Causation	Probably 2 viruses, that of Strain 2 and a virus of osteoblastoma	1 virus	1 virus
Gross appearance of tumors	Soft with hard areas of bone and cartilage	Soft or pulpy, with clefts containing hemorrhage	Tough
Rate of growth	Variable, usually slow	Rapid	Very slow
Characteristic malignant cell	Large round or polygonal cells (osteoblasts)	Peculiar polymorphous giant cells	Spindle, polymorphous and mononuclear cells; occasional giant cells

* This investigation has been supported by a Fund for the Study of Leukemia Mr. Charles Breedis assisted in the work.

Characteristics of Strain 15

Origin.—On Mar. 27, 1934, a Barred Rock chick (No. 4534) weighing 180 gm., was anesthetized with ether and injected into the exposed right sciatic nerve with plasma of a chicken with neurolymphomatosis of Strain 6 (5). On Aug. 3, 1934, there was weakness of the right leg and in the belief that it had paralysis 3 chickens were injected intravenously on Aug. 16, each with 0.3 cc. of its blood. The chicken was killed on the following day and a tumor was found in the right pelvis replacing the greater part of the kidney. The liver contained many minute (1 to 2 mm.) tumor nodules. There was no evidence of leukosis or neurolymphomatosis. Particles of the tumor kept overnight in the ice box were injected into 2 chickens, both intramuscularly and intraperitoneally (Table I).

TABLE I

Injections of Tumor Tissue and Blood of Tumor-Bearing Chickens (Strain 15)

No. of passage	Material injected	Route of injection	No. of chickens injected	No. of successful injections
I	Blood	i.v.	3	0
	Tumor	i.m. and i.p.	2	1
	Blood	i.m.	2	0
II	"	Intraneural	3	0
	Tumor	i.m.	14	14
III a	"	"	3	3
III b	Blood	i.v. and i.m.	3	0
IV a	Tumor	i.m.	4	4
IV b	"	"	4	4
IV c	"	"	3	3
Total	Blood		11	0
	Tumor		30	29

Route of inoculation: i.v. = intravenous, i.m. = intramuscular, s.c. = subcutaneous.

Results of Transmission Experiments.—The 3 chickens injected with the blood of No. 4534 remained healthy, but 1 of the 2 chickens injected intramuscularly with tumor tissue developed a rapidly growing tumor in the injected breast muscle. The other, killed 71 days after injection, showed no evidence of leukosis or tumor. Subsequent intramuscular inoculations with tumor tissue, summarized in Table I, were successful in every chicken injected, but the virus of this sarcoma was not recovered from the blood.

These experiments show that even though the chicken from which Strain 15 was derived had been injected with plasma of a fowl with

neurolymphomatosis, the tumor material and blood do not produce neurolymphomatosis.

Inoculations with material not containing live cells, summarized in Table II, show that the disease is readily transmitted with Berkefeld filtrate of tumor extract, desiccated tumor, and extract of dried tumor.

The size of these cells varies from approximately 9 to 25 micra in longest diameter. Their cytoplasm is pale and contains one or two eccentrically located nuclei. The cytoplasm of many cells is ill defined but the nuclei are sharply outlined. They are very clear, appear to be almost devoid of chromatin, and contain one or two nucleoli that stain dark blue or red.

TABLE II

Inoculation with Material Free from Viable Cells (Strain 15)

No. of passage	Material injected	Route of injection	No. of chickens injected	No. of successful injections	Control	
					No. of chickens injected	No. of successful injections
II	Dried tumor particles	i.m.	4	4	14	14
IIIa	Tumor filtrate	i.v. and i.m.	3	3	—	—
	Dried tumor particles	i.m.	4	3	3	3
	Dried tumor extract	i.m. and s.c.	8	5	—	—
IIIc	Dried tumor particles	i.m.	4	3	—	—
	Extract of 3 dried tumors	i.v.	4	3	—	—
Total.....			27	21	17	17

The control injections were made with fresh tumor tissue. A medium Mandler filter was used in the second experiment with an air bubbling pressure of 33 cm. Hg and a flow of water of 28 cc. per minute at 40 cm. Hg.

The metastatic tumors are usually formed by giant cells as shown in Figs. 5 to 8, but in the primary tumors, and occasionally in tumors of internal organs, there are many spindle-shaped cells that are indistinguishable from those of common sarcomata (Figs. 2 and 4).

The origin of the predominant cells of Sarcoma 15 has not been determined. Silver stained preparations[1] did not show nerve fibers.

[1] Studied by Dr. Lewis D. Stevenson.

The readiness with which the tumor is produced by the virus in the areolar tissue of the wing makes it unlikely that it is derived from muscle elements; the absence of the virus from the blood as judged by the results of the experiments shown in Table I may mean that it is not a neoplasm of macrophages. Microscopic sections (Fig. 7) indicate that the tumor cells nevertheless invade the blood and occasionally show transitional forms between cells like monocytes and tumor giant cells.

Anatomical Characteristics.—Some of the characteristics of the neoplasm produced by this strain have already been stated in the introduction to this paper and compared with those of Strains 11 and 12. The tumors grow rapidly in muscle tissue as well as in the subcutaneous areolar tissue of the wing. They are gray, soft, fleshy masses split into several parts by sinuses containing bloody, viscous material (Fig. 1). Absence of bone or cartilage differentiates these tumors from those of Strain 12, and they are never tough like the sarcomata of Strain 11. When the injection is made through the sternal keel, tumor tissue may infiltrate the marrow cavity, but in most instances separate tumors are formed on either side of the sternal keel.

Of the 39 chickens with tumors at the site of inoculation, metastases were found in 21; but with one exception none of the inoculated chickens lived longer than 62 days. The lungs were the most frequent site of metastasis (found in 20 chickens), and in many chickens the greater part of this organ was replaced by tumor. The metastases in liver (found in 8 chickens) were less frequent and less extensive. Tumors in the heart, kidney, or spleen were found in each of 3 or 4 chickens.

The diagnostic cells of this strain are illustrated in Figs. 5 to 8. Wherever found they can readily be distinguished from the predominant cells of other chicken tumors that are caused by filterable viruses.

Characteristics of Strain 11

The virus of Strain 11 has distinctive features that differ from those of the three other sarcomata observed by us (3, 4), but is similar to the spindle cell sarcoma of Rous (2, 1). All chickens inoculated with it were kept in one room, often in one cage, together with chickens inoculated with our different agents of leukosis and sarcoma. In the course of the passages, however, leukosis or neoplastic alterations, such as are produced by sarcoma Strains 12 and 13, were not observed.

Origin.—Strain 11 originated in an uninoculated Barred Rock chicken (No. 3264) received at the age of approximately 4 months (November, 1932). It was kept in a cage with 3 chickens inoculated with Strain 2. 4 months after the bird

was received, many firm gray tumor nodules were found in its skin, measuring from 0.5 to 2 cm. in diameter. Similar tumor nodules were found in the subcutaneous tissue and liver. There was no evidence of leukosis.

Transmission Experiments.—The chicken was killed, tumor tissue cut up in the presence of Locke's solution, and tumor particles injected into the skin, subcutaneous tissue, and breast muscle of 4 chickens. Similar material was filtered

TABLE III

Injections of Fresh Tumor Tissue of Strain 11

No. of passage	Route of injection	Total No. of injections	No. of successful injections
I	i.v.	2	1
	i.m.	4	3
II a	"	3	2
III	"	8	7
IV	i.v.	3	1
	i.m.	3	2
V	"	3	3
VI	i.v.	3	1
	i.m.	2	2
VII	"	3	3
VIII a	"	3	2
VIII b	"	3	2
Total.....	i.v.	8	3 (37.5%)
	i.m.	32	26 (81.3%)

The tumor produced regressed in 4 of the 7 chickens of passage III. For intravenous injection the tumor cell suspension was filtered through cotton.

through cotton and injected intravenously into 2 chickens. 3 of the 4 chickens developed tumors at the site of injection (Table III), the fourth remained healthy and was given another injection of tumor particles in the breast muscle 5 months later. A slowly growing fibrosarcoma developed at the site of reinjection.

The results of inoculations with tumor tissue deriving from chicken 3264 are summarized in Table III. Fresh tumor tissue injected in-

tramuscularly produced tumors in 81 per cent, that injected intravenously in 37.5 per cent of the inoculated birds. Of the injections made with tumor tissue, dried in the frozen state, 54 per cent were successful (Table IV).

Anatomical Characteristics.—The tumors of Strain 11 are slow growing, tough, confluent, nodular masses of tissue with microscopic appearances illustrated in

TABLE IV

Intramuscular Inoculations with Desiccated Tumor Tissue of Strain 11

Desiccated tumor				Fresh tumor	
No. of chicken (donor)	Age of material injected	Total No. of injections	No. of successful injections	Total No. of injections	No. of successful injections
	days				
3593	3	3	3	—	—
3324	11	3	0	6	3
	27	2	2		
3705	54	2	0	—	—
3842	49	2	2	—	—
4391	271	4	1	3	3
3533	456	4	1	—	—
4167	319	4	3	—	—
4820	162	4	3	—	—
Total.....		28	15	9	6

From 1 to 4 four samples were tested on each chicken and each sample was tested on from 2 to 4 chickens.

Figs. 9 to 12. The chronicity of these tumors is indicated by the small number of passages that have been required to keep this strain alive during 2 years.

Of 17 chickens injected intramuscularly, metastases were found in 6, of which 3 were killed from 55 to 172 days, and 3 died from 45 to 210 days, after injection. The size of the primary tumors found in the muscle tissue at the time of death varied from 1.5 x 1 x 1 cm. (53 days after injection) to 7 x 4 x 4 cm. (172 days after injection). The primary tumors in the chickens which had no metastases were of similar size and the birds died or were killed from 43 to 136 days after inoculation.

To determine the favorite sites of growth of Sarcoma 11, 13 chickens were injected intravenously. These birds died after from 43 to 183 days with the exception of one that was killed after 61 days. Thus 19 chickens were available in the study of the localization of lesions in sites other than the injected muscle tissue. Tumors were found in the liver (13 cases), heart (8 cases), lung and spleen (5 cases each), voluntary muscle and kidney (4 cases each), and in only one or two cases in the following organs: thymus, mesentery, adrenal, ovary, skin, bone, or bone marrow.

Combined Inoculation with a Pure Leukosis and a Pure Sarcoma Virus

The following experiments were undertaken to obtain information on the character of the naturally occurring strains that produce both sarcoma and erythroleukosis. Such strains have been recently described by several workers (6, 7, 4) and the question arose whether they are produced by one or several viruses. In Experiments A and B the virus of pure sarcoma (Strain 11) and the virus of pure leukosis (Strain 1) were injected separately into the same chickens.

Experiment A.—3 chickens received an intravenous injection of 0.2 cc. of leukotic blood of Strain 1 and a similar injection of 1 cc. of tumor extract of Strain 11. One died 33 days after injection with a 7 mm. tumor at the site of intravenous needle puncture and two 2 mm. tumor nodules in the spleen. Another died 116 days after injection showing numerous tumor nodules in the subcutaneous and muscle tissues and liver and a pathological fracture of the right femur. These 2 chickens gave no evidence of leukosis; but the third died of erythroleukosis combined with myeloid leukemia 59 days after injection, showing no evidence of sarcoma.

Experiment B.—Splenic cell suspension of a chicken with erythroleukosis (Strain 1) was injected intramuscularly and intravenously into each of 4 chickens. 3 days later sarcoma particles (Strain 11) were injected intramuscularly into the same chickens. One killed 141 days and another one that died 109 days after the first injection developed sarcoma only. The third, killed after 30 days, had sarcoma and erythroleukosis, and the fourth, killed after 26 days had sarcoma and aplastic anemia.

Microscopic examinations have shown that one virus did not modify the effect of the other virus and that the histological changes produced by the complex Strain 13 (4) were not reproduced by the combination of sarcoma of Strain 11 and leukosis of Strain 1. Therefore, if Strain 13 is composed of a mixture of sarcoma and leukosis viruses

the sarcoma virus is different from that of Strain 11. The experiments show furthermore that *in vivo*, Sarcoma 11 and leukosis do not help each other.

Inoculations were made with the blood of chicken 4966 (of Experiment B) that had sarcoma and aplastic anemia with the purpose of reisolating leukosis of Strain 1 free from sarcoma. Previous studies have shown that the virus of erythroleukosis may give rise to chronic anemia with no evidence of erythroblastic proliferation (8), that is, to an alteration which is doubtless not neoplastic. The agent of leukosis of Strain 1 circulates in the blood in very high concentration (9) but the virus of sarcoma is often absent in the blood of tumor-bearing chickens (see for example the experiments given in Table I).

Experiment C.—The hematocrit values of the blood of chicken 4966 were: plasma, 94.8 per cent; erythrocytes, 5 per cent; and leukocytes, less than 0.2 per cent. The blood smear showed an occasional polychrome erythrocyte and that most of the white cells were small lymphocytes. The bone marrow contained abundant fat and showed only very slight evidence of blood-forming activity. 4 chickens were injected each with 0.5 cc. of blood of this chicken, intravenously, with 0.3 cc. intramuscularly. 3 of the 4 chickens developed characteristic erythroleukosis, but none had sarcoma.

Thus transmission with blood from a chicken that had aplastic anemia (produced by Strain 1) and sarcoma (produced by Strain 11) gave rise to erythroleukosis only. Experiment C shows that when a chicken has erythroleukosis and sarcoma produced by two viruses, the virus of erythroleukosis may be readily reisolated from the blood which often does not contain the virus of sarcoma (3).

In the following experiment an attempt was made to reisolate the virus of sarcoma from a chicken that had both erythroleukosis and sarcoma.

Experiment D.—Tissue cultures were prepared from chicken 4963 (of Experiment B) that had both sarcoma and leukosis. The tumor particles were embedded in the homologous plasma over which serum of the same chicken was placed. The cultures consisted of spindle-shaped and fibroblast-like cells and there was no evidence that they were related to monocytes or to histiocytes.

Subcultures were made at from 4 to 6 day intervals in plasma and serum of normal chickens. The results of inoculations with these tissue cultures were as follows:

Age of culture	No. of subculture	No. of chickens injected	No. with sarcoma
days			
Immediate	—	4	4
9	I	4	3
17	II, III	4	4
23	IV	6	6
33	VI, VII	2	2

It has not been possible thus far to keep the virus of erythroleukosis alive *in vitro* for more than a few days. Hence it may be expected that tissue cultures of sarcoma from chickens that have also leukosis will yield the pure virus of sarcoma. Experiment D shows that all birds inoculated with the tissue cultures developed sarcoma and none had leukosis, with the exception of one chicken, accidentally killed 9 days after injection, which appeared healthy. This is in striking contrast to the findings with sarcoma of Strain 13, a pure culture of which produces both sarcoma and erythroleukosis (10). These experiments indicate that the sarcoma cells of Strain 11 do not support the growth of the virus of leukosis of Strain 1.

Caldwell (11) distinguishes four types of interaction of two strains of plant viruses: (*a*) a virus may inhibit the development of another virus in the host tissues; (*b*) the second virus may multiply in the tissue without inducing typical disease symptoms; (*c*) the two viruses may multiply each inducing typical symptoms of its disease; (*d*) the effect of the second virus may cause a more severe disease than either virus alone could have caused. There are no experimental data available concerning the interaction of tumor-producing viruses. The experiments here described show that leukosis of Strain 1 and sarcoma of Strain 11, when injected into the same animals, retain their identity and can be reisolated. These experiments do not elucidate the behavior of some complex transmissible strains (*e.g.* endothelioma and erythroblastic proliferation seem to be produced by a single virus of Strain 13 (4)), but they demonstrate that viruses of sarcoma and leukosis retain their identity under varied experimental conditions.

SUMMARY AND CONCLUSIONS

Two transmissible sarcomata (Nos. 15 and 11) are described. Strain 15 originated in a chicken injected with neurolymphomatosis

the sarcoma virus is different from that of Strain 11. The experiments show furthermore that *in vivo*, Sarcoma 11 and leukosis do not help each other.

Inoculations were made with the blood of chicken 4966 (of Experiment B) that had sarcoma and aplastic anemia with the purpose of reisolating leukosis of Strain 1 free from sarcoma. Previous studies have shown that the virus of erythroleukosis may give rise to chronic anemia with no evidence of erythroblastic proliferation (8), that is, to an alteration which is doubtless not neoplastic. The agent of leukosis of Strain 1 circulates in the blood in very high concentration (9) but the virus of sarcoma is often absent in the blood of tumor-bearing chickens (see for example the experiments given in Table I).

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Thus transmission with blood from a chicken that had aplastic anemia (produced by Strain 1) and sarcoma (produced by Strain 11) gave rise to erythroleukosis only. Experiment C shows that when a chicken has erythroleukosis and sarcoma produced by two viruses, the virus of erythroleukosis may be readily reisolated from the blood which often does not contain the virus of sarcoma (3).

In the following experiment an attempt was made to reisolate the virus of sarcoma from a chicken that had both erythroleukosis and sarcoma.

Experiment D.—Tissue cultures were prepared from chicken 4963 (of Experiment B) that had both sarcoma and leukosis. The tumor particles were embedded in the homologous plasma over which serum of the same chicken was placed. The cultures consisted of spindle-shaped and fibroblast-like cells and there was no evidence that they were related to monocytes or to histiocytes.

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SUMMARY AND CONCLUSIONS

Two transmissible sarcomata (Nos. 15 and 11) are described. Strain 15 originated in a chicken injected with neurolymphomatosis

of Strain 5. Its virus produces sarcoma characterized by peculiar giant cells, different from those of the other known chicken sarcomata. It does not produce neurolymphomatosis or leukosis.

Strain 11 is morphologically similar to the spindle cell sarcoma of Rous and of other workers. It occurred in an uninjected control chicken and its virus produces sarcoma unassociated with leukosis.

Both Strains 11 and 15 can be readily transmitted with material free from viable cells and their virus can be preserved by drying in the frozen state.

The viruses of leukosis of Strain 1 and sarcoma of Strain 11 when injected into the same bird retain their identity and can be readily reisolated.

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EXPLANATION OF PLATES

The sections were stained with hematoxylin and eosin-azure solutions. The magnifications given are only approximate.

PLATE 11

FIG. 1. Huge, soft hemorrhagic tumors on both sides of the sternal keel produced by intramuscular injections of saline extract of desiccated tumor (Strain 15). The chicken died 41 days after injection. There were extensive metastases to the lungs and liver.

FIGS. 2 and 3. Microscopic appearance of the tumors produced in the breast muscle with dried tumor particles (Strain 15). Magnifications: Fig. 2, × 120; Fig. 3, × 600.

FIG. 4. Sarcomatous infiltration of the sternal keel. The chicken died 78 days after an intravenous injection of saline extract of desiccated tumor (Strain 15). × 180.

PLATE 12

FIG. 5. Sarcoma in the lung, composed of the giant cells characteristic of Strain 15. The chicken died 78 days after an intravenous injection of saline extract of desiccated tumor. × 250.

FIG. 6. Sarcoma in the spleen, composed of the giant cells characteristic of Strain 15. The chicken died 39 days after intramuscular injections of dried tumor particles. × 150.

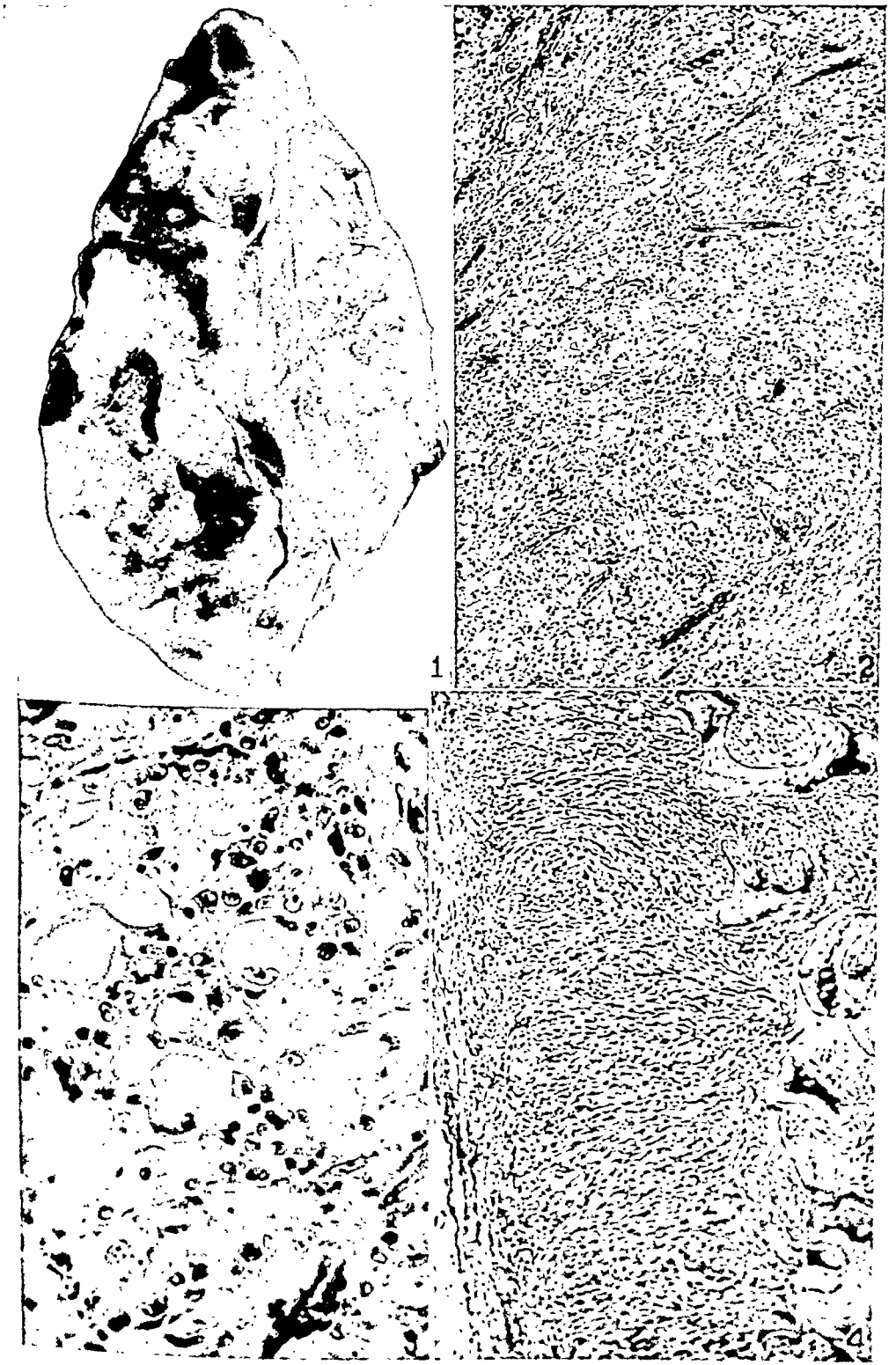
FIG. 7. Sarcoma in the liver (Strain 15). There are many tumor cells free in the blood vessel. × 200.

FIG. 8. Higher magnification (× 600) of Fig. 7. The liver cells are dark stained, the tumor giant cells are light.

PLATE 13

FIGS. 9 to 11. The tumor cells of Strain 11. Figs. 10 and 11 are from the spontaneous tumor. Fig. 9 is from a tumor produced by intramuscular implantation of the spontaneous growth (first passage). × 450.

FIG. 12. Sarcoma in the kidney. The chicken was injected intramuscularly with fragments of a 33 day old tissue culture of Sarcoma 11 and intravenously with the supernatant liquid of the culture. (See Experiment D.) × 450.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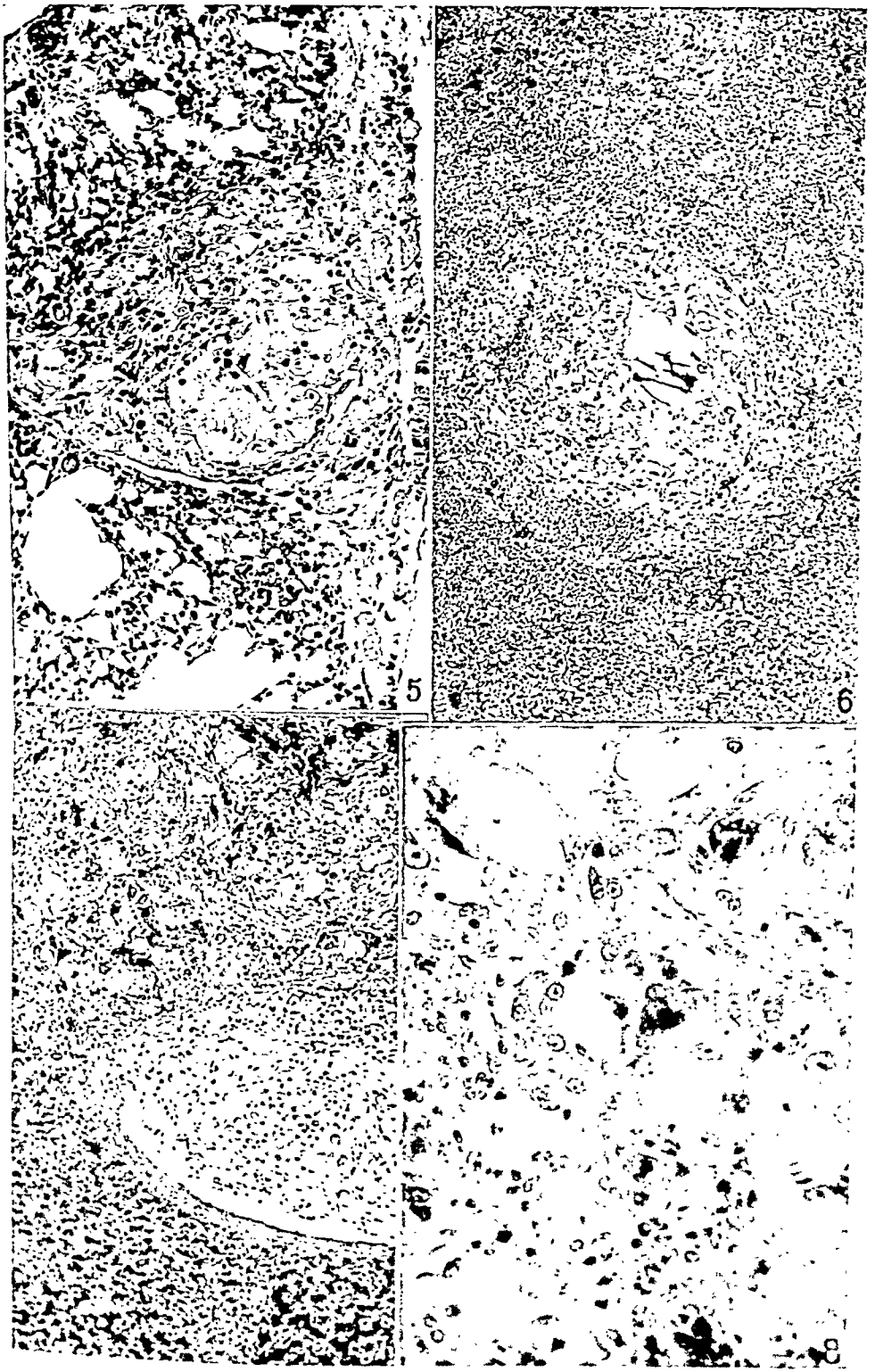
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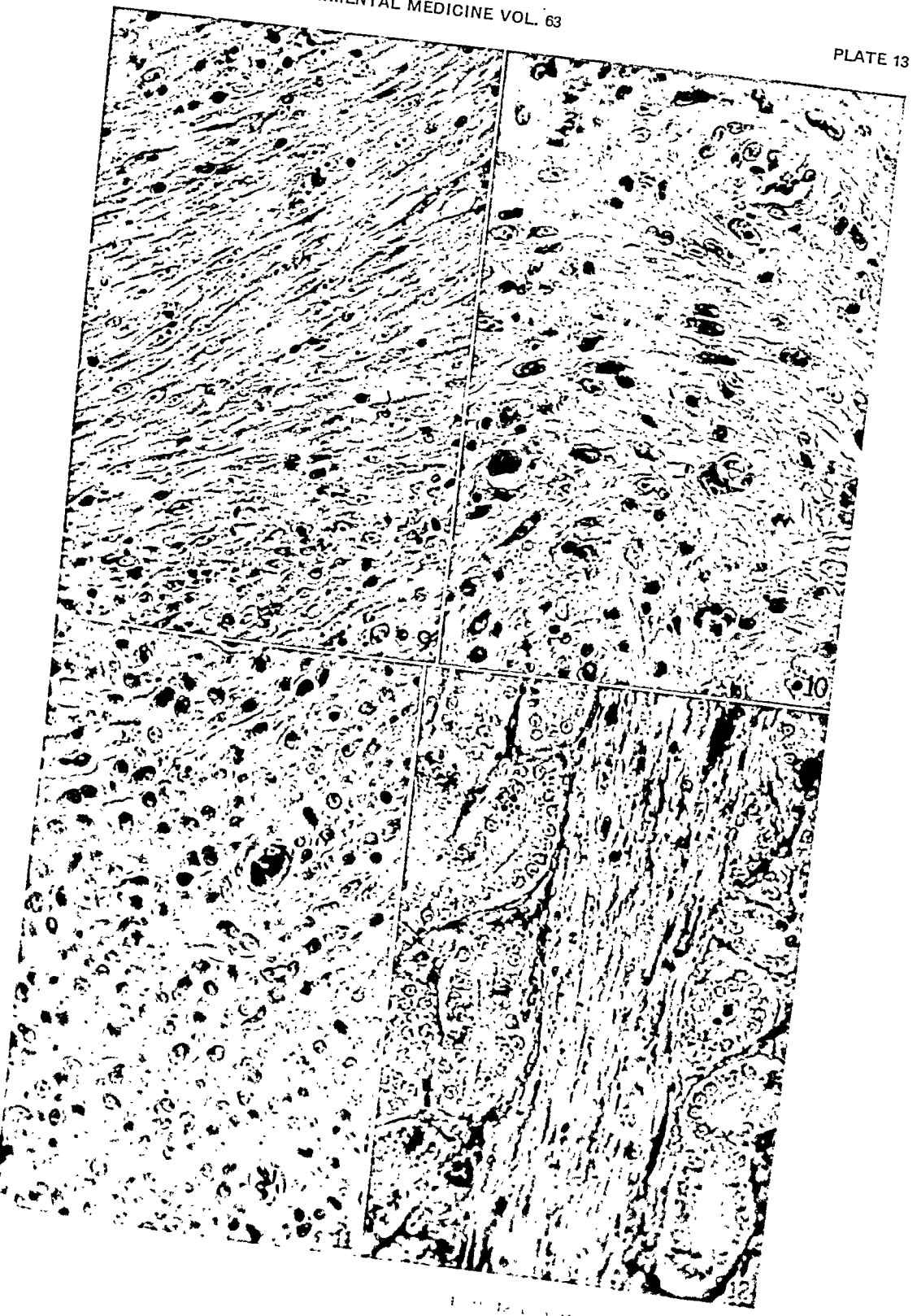
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A CHANGE IN RABBIT FIBROMA VIRUS SUGGESTING MUTATION

I. Experiments on Domestic Rabbits

By C. H. ANDREWES, M.D.

(*From the National Institute for Medical Research, Hampstead, London*)

Plate 14

(Received for publication, October 23, 1935)

In 1932 Shope (1) described an infectious fibroma of the cottontail rabbit. This proved to be caused by a filtrable virus which would infect not only cottontails but also domestic rabbits. On inoculation into the testis, skin or muscle, the virus produced localized tumour-like swellings, apparently formed from multiplication of young connective tissue cells. The growths did not metastasize and ultimately always regressed, more quickly in the domestic rabbit than in the cottontail. Few, if any, pathologists would regard them as true tumours.

In this paper I have described observations made in England on the behaviour of strains of virus sent to me by Shope. He has recorded, in the succeeding paper (2), the results of parallel observations made by himself at Princeton. In the third paper of the series (3), we have jointly discussed the interpretation of our findings. A certain amount of repetition and cross-reference in the first two papers is unavoidable.

In February, 1933, I received from Shope glycerolated material from two strains of fibroma virus, A and B. One of them, Strain A, was that studied in Shope's original experiments; yet it behaved from the moment I received it in a manner quite different from that described by him. It no longer gave rise to fibroma-like growths, but produced only acute inflammatory lesions; furthermore, many inoculated rabbits developed a generalised pock-like eruption on the skin. In the succeeding paper (2), Shope describes the history of the A strain of virus before he supplied me with material, and also records a similar but less extensive change which the virus underwent in his laboratory.

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In November, 1933, Shope kindly gave me material of his A strain which had been preserved in glycerol for 18 months and dated back to a time before the first appearance of any inflammatory traits. In my hands this strain has continued on passage to produce fibroma-like lesions such as Shope originally described. I have thus been able to compare the two strains side by side; one is referred to as the original A (OA), the other as the inflammatory A (IA) strain. Evidence will be presented that the two cross-immunise, one against the other, and that the IA does not represent a contamination with any other known virus.

Behaviour of the OA Strain

The original A strain behaved, in my hands, mainly according to Shope's description (1). In rabbits killed a week or less after intratesticular inoculation, the naked eye appearance of the testis was that of an acute inflammation rather than tumour formation; there was scrotal oedema and the testis was engorged and exuded fluid on section. Later than this, the formation of large fibroma-like masses was more and more evident; these persisted for a week or two before regressing. In some animals, however, the testis became impacted in the inguinal canal in the course of its enlargement and then haemorrhagic necrosis set in, instead of further increase in size.

In one respect my experiences differ from those described by Shope; subcutaneous inoculation gave inconstant results, but intradermal injection of 0.1 cc. of virus regularly produced sharply circumscribed raised red lesions. These appeared in 2 or 3 days and grew until about the 12th or 14th day, often reaching a diameter of 4 or 5 cm. and a thickness of more than 1 cm. The skin over them often had a vesiculated appearance. After 12 or 14 days regression began, with or without central necrosis of the skin overlying the lesion. Small satellite lesions were not infrequently seen. Suspensions of affected testes often produced skin lesions up to a dilution of 1:10,000 or 1:100,000 of the original 10 per cent testicular emulsion, and a convenient method of titrating the virus was thus available.

Histology of OA Lesions

Testes removed 3 to 6 days after inoculation often showed histologically hyperaemia, local oedema of the interstitial tissues and some exudation of polymorphonuclear cells. Later one could see that large mononuclear cells were present in increased numbers and soon the whole picture was dominated by the presence of large numbers of young fibroblast-like cells, showing numerous mitotic figures (see Fig. 1). The fibroma-like tissue thus formed continued to increase for some days and apparently to become more mature and less cellular as it did so. In the midst of this older fibromatous tissue, blood vessels could often be seen surrounded by a more cellular zone. The period at which these changes occurred

was inconstant; the dose of virus used was an important factor. Thus, testes removed after 7 days showed sometimes only the earlier exudative stage, but more usually the fibroblastic proliferation had already made considerable progress. In some testes the proliferative reaction was found to have set in within a few days of inoculation and no trace was evident that there had ever been any oedema or exudation of polymorphonuclear leucocytes. Except where local necrosis occurred, signs of inflammation regularly became less as the fibroblastic proliferation continued, but often oedema, hyperaemia and polymorphonuclears were evident even in the later lesions in the visceral layer of the tunica vaginalis, around the epididymis and in the fat in the spermatic cord. Necrosis of the germinal epithelium was often present a week after inoculation, but all the evidence suggests that it was secondary to the changes in the interstitial tissues.

Histological studies of the skin lesions were less extensive. The most prominent feature at first was the oedema of the subepidermal fibrous tissue; mononuclear infiltration occurred around the hair follicles and the deeper blood vessels. Later, fibroblastic proliferation began in the oedematous connective tissue near these vessels outside the panniculus carnosus. The fully developed growths both in skin and testis showed the changes described by Shope (1), being almost entirely composed of masses of fibroblast-like cells arranged in sheets, strands or whorls.

Behaviour of the IA Strain

The early changes produced by the two strains of virus are apparently identical. A few days after intratesticular inoculation with the IA strain the testes on palpation are full and firm and there may or may not be scrotal oedema. When testes from rabbits killed less than a week after inoculation are examined, it is often impossible to tell with the naked eye which strain is concerned: in both instances there is evidence of acute orchitis. Similarly the skin lesions produced by the IA strain are at first like those already described. Rarely, however, are they as definitely raised and circumscribed as the OA lesions. It is in the later behaviour of its testicular and skin lesions, that the IA strain shows itself to be so different. After a week the acute orchitis subsides, but the testis, instead of progressively enlarging, rapidly begins to atrophy and a fortnight from inoculation it is already much smaller than normal. The raised red areas on the skin also begin to decrease in size on the 6th or 7th day and central necrosis is then the rule. The skin lesions produced by both strains have been measured daily and the changes in their si plotted graphically; it has then been very striking to see how those of the OA vir continue to grow after the 6th or 7th day, though more slowly than at first, whi those of the IA strain rapidly resolve and have often disappeared at a time whe comparable OA skin growths are still progressing.

Histology of IA Lesions

Histologically, also, the two strains produce at first similar changes. But with the IA virus the early oedema and polymorphonuclear infiltration soon give

place to massive accumulation of lymphocytes in the interstitial tissues (Fig. 2). This is accompanied by atrophy of the parenchyma. After a week fibrosis and atrophy of the testis as a whole supervene. The fibrosis is such as will follow almost any acute orchitis and cannot readily be confused with the vast fibromatous proliferation produced by the other strain. In the early stages a number of large cells, possibly histiocytes, are sometimes seen in the testicular interstitial tissues, apparently dying as the result of the infection; they are swollen and have flattened crescentic nuclei pushed to the periphery of the cell.

The IA skin lesions are like those of the other type in all their early stages. The later fibromatous proliferation is, however, absent and the prompt regression of the lesion is accompanied by copious accumulation of lymphocytes.

Generalised "Pocks" Produced by the Rabbit Fibroma Virus

At the 6th serial rabbit passage after I had received from Shope the IA strain, I first noted the appearance in the skin of small red papules strongly suggestive of the early lesions of generalised vaccinia in the rabbit. Subsequently, between April, 1933, and October, 1934, the eruption was seen in 43 out of 80 inoculated rabbits (54 per cent) in which the hair had been clipped and the skin observed for 7 days or more. The rash appeared usually on the 6th, 7th or 8th day after inoculation. The papules were raised, red, 2 to 5 mm. in diameter, occasionally larger; they grew for a day or two at most and then regressed without vesiculation or pustulation. They appeared after intratesticular or intradermal inoculation and could be seen on the skin of a flank on which the hair had been clipped but which had not been itself inoculated. Papules were not seen on the ears or mucous membranes. Virus was recovered from the papules by excising them, grinding them up and inoculating a suspension into another rabbit. I failed to recover virus from areas of skin not showing papules, nor in two attempts did I obtain any from the blood of an infected rabbit. Not only was whole blood used in these two experiments, but also, in view of Smith's work with vaccinia (4), washed blood cells.

Generalisation of the virus to the skin was at first thought to be peculiar to the IA strain of virus. Though it was not noted until the 6th passage of this strain at Hampstead, in only 2 rabbits of earlier passages had the hair been clipped; an eruption would thus probably have been unobserved. In experiments with the OA virus no generalisation was seen during the first 7 passages; at this stage 22 rabbits had been clipped and watched for the appearance of an eruption, with

negative results. Generalisation was first seen at the 11th passage and thereafter it appeared frequently. Otherwise, the virus behaved as before and showed no tendency to change to the IA type. When generalisation unexpectedly appeared in the OA series, I went back to glycerolated rabbit testis of the 6th, 8th and 9th passages, which had been kept in the cold room, and proceeded to make serial transfers from them. In 4 different series, skin eruptions appeared in rabbits inoculated with virus of 8th, 9th, 10th and 11th passage respectively. In 33 rabbits of the 7th and subsequent passages generalisation occurred in 9, or 27 per cent. The pocks were like those of the IA strain and showed, like them, no tendency to progressive increase in size. Histologically, also, the secondary lesions produced by the two strains were alike; there were no constant changes other than local oedema and round celled infiltration. Virus recovered from pocks of OA rabbits proved on further inoculation always to be of the OA type.

An unexplained feature of the generalisation is this: it has regularly occurred in my hands, with both strains and in rabbits of several different breeds including Dutch, Chinchilla, Himalayan, Belgian hares and half-lops; yet Shope (2) has never noted it with either virus strain in America, though he has watched for it carefully. He has worked with all the breeds of rabbits mentioned, except the half-lops. The conditions determining generalisation are thus obscure. As already mentioned, it occurred in 54 per cent of my rabbits over a period of 18 months. After that it was seen much less commonly, between October, 1934, and May, 1935, only in 12 out of 74 inoculated rabbits (16 per cent).

"*Inclusion Bodies*"

Shope (1) reported that the fibroma virus produced in the cottontail rabbit skin lesions recalling those of molluscum contagiosum in man. Masses of epithelial cells projected downwards and many cells were enlarged and contained eosinophilic masses in their cytoplasm. He found, however, no "inclusions" in the lesions in domestic rabbits. I, too, have failed in the large majority of sections of tame rabbit material examined to find any inclusion bodies, nor have I ever seen changes as striking as those found in cottontails. I have,

however, found in the cytoplasm of epithelial cells in two sections of skin, eosinophilic masses recalling the cottontail inclusions. One of these was in a rabbit infected with the IA strain, the other in an OA rabbit. In the epididymis, also, I have found on two occasions areas of considerable epithelial proliferation. In one rabbit, infected with OA virus 7 days before, this proliferation entirely occupied the lumen of many tubules; the epithelium was degenerate and inclusions could not be recognized with certainty. In the other rabbit, inoculated 10 days previously with the IA strain, the proliferation was less striking but eosinophilic inclusions were visible in the cytoplasm of many epithelial cells in the tubules. These few observations suggest, particularly in view of the changes it produces in the cottontail, that virus of both strains is potentially epitheliotropic in the domestic rabbit.

Cross-Immunity between OA and IA Strains

Rabbits recovered from infection with one strain were found to be immune to inoculations of the other. The existence of cross-immunity was shown by the absence of lesions on reinoculation into skin or testis or the occurrence at the site of an intradermal inoculation of an accelerated allergic type of response reaching its maximum in 48 hours. Accelerated reactions of this type occurred just as frequently when the test was made with the homologous strain as in cross-immunity tests. 17 rabbits inoculated into the skin with IA virus were immune when tested after 14 to 38 days with OA virus. 5 other IA rabbits were similarly immune when tested 7 to 37 days later with another strain of virus, B, which at that time was behaving in all respects like the OA strain. Conversely, 8 rabbits recovered from OA infections were found resistant to the IA virus in tests made after 14 to 24 days.

Neutralisation tests with immune rabbit sera confirmed this result. It was not even possible to demonstrate quantitative differences in neutralising power such as can be shown when antisera against various fowl tumour viruses are carefully compared with each other. Undiluted OA virus when mixed with an equal volume of 1 in 5 antiserum and held for an hour at room temperature was not neutralised either by homologous (OA) or by IA antiserum; 1 in 5 dilutions of both sera

were, however, effective against the same virus diluted 1 in 10. In tests against IA virus, neither serum in a 1 in 5 dilution was able to neutralise completely even the 1 in 100 dilution of virus; but undiluted sera from both OA- and IA-immune rabbits completely inactivated undiluted virus.

Tests for Cross-Immunity between IA Virus and Other Viruses

When first the IA virus began to behave in an unexpected manner and particularly when generalised pocks were seen, it was suspected that the material under study might have become contaminated with vaccinia or some other virus. It was found, however, that a rabbit recovered from infection with IA virus was, when tested 25 days later, fully susceptible to intradermal inoculation with neurovaccinia virus. Further, the IA virus was not in the smallest degree neutralised by an antivaccinial serum of proved activity.

Dr. Louise Pearce kindly gave me two sera from rabbits recovered from the rabbit pox studied at The Rockefeller Institute (5); neither had any neutralising power against IA virus. The virus was also unaffected by antiserum active against Virus III of Rivers and Tillett (6).

A few experiments were carried out bearing on a possible relationship between the rabbit fibroma and a true tumour of the rabbit. 14 rabbits in which Brown-Pearce tumours had regressed were taken; 7 of them were hyperimmunised with 1 to 6 subcutaneous injections of cells from the same tumour and then all were tested intradermally with fibroma virus (IA) along with control rabbits. The tumour-immune rabbits all proved fully susceptible. Sera obtained from 7 of the tumour rabbits before this intradermal test were found to be devoid of neutralising power for IA virus.

Four antisera active against fowl tumour viruses (Rous No. 1, Fujinami, RF4 and CT10) (7) were tested and found to be unable to neutralise IA virus. Experiments on cross-immunity with rabbit myxoma virus are reported in the following paper (2).

Attempts to Convert One Strain into the Other

The available evidence suggested that the IA strain was not a contamination with any other known virus, but that it and the OA

strain were varieties of one virus, possibly differing in their virulence for the cell. The following experiments were planned with this as a working hypothesis.

IA virus was passaged at approximately weekly intervals from testis to testis through 28 rabbits in series; it showed no tendency to revert to the fibroma-producing phase. After this, transfers were made at fortnightly intervals for 3 passages. Very little IA virus is recoverable from a testis after 14 days and it was thus hoped to attenuate it or decrease its virulence. Although virus was not readily carried on by passages at these longer intervals, no tendency was seen after 4 such transfers for the virus to produce fibromatous changes or otherwise change its character. An attempt was made to lessen the vigour of the reaction to the virus by lowering the temperature at which the reaction occurred: inoculations were accordingly made intradermally into the ear of a half-lop rabbit, but the type of lesion was not essentially modified.

Next, injections of IA virus were made into a rabbit's left testis a few days after a primary inoculation into its right testis; it was thought that if the cells should possess a small degree of active immunity they might react less violently and produce an OA type of lesion. This hope was not realised; when the interval between the injections was 2 days, the second inoculation produced a reaction of the usual type; the same was seen after a 4 day period except that lymphocytic infiltration was more than usually prominent. When the interval was extended to 6 days, the second testis was apparently immune, as it appeared normal microscopically. In yet other experiments, incomplete neutralisation of IA virus with antiserum failed to influence the type of reaction produced by it.

A similar lack of success attended efforts to enhance the virulence of the OA virus so as to cause it to produce only an inflammatory tissue response. After 14 rapid passages at approximately weekly intervals its behaviour was in this respect unchanged; it had, however, as already recorded, apparently acquired the power of producing a generalised skin eruption.

Shope's "Changed Virus"

In October, 1933, Shope gave me a glycerolated virus in rabbit testis with the following history: it was derived from virus which produced predominantly but not entirely inflammatory reactions and is described by him as changed virus (2); this, as he has recorded, was inoculated into a cottontail rabbit, and the virus recovered therefrom was found to have regained temporarily its fibroma-producing properties. When I inoculated this into rabbits, only fibromatous (OA) lesions were seen in the first animal infected, but passage from that rabbit's testis produced, in four rabbits, predominantly inflammatory

lesions. From the 2nd passage onwards, generalisation was frequent. Testes of 8 rabbits of the 2nd to 4th passages, when killed after 7 to 31 days, showed remarkable changes, strongly suggesting a mixture of the effects produced by the OA and IA strains. When such testes were sliced in half, firm white nodules could be seen in the midst of softer hyperaemic tissue. The impression gained was confirmed by histological study. Through the greater part of the testis could be seen severe inflammatory reaction in the interstitial tissues; in most sections lymphocytes predominated. In the midst of this inflamed tissue were sharply circumscribed fibromatous nodules, often with the proliferating cells arranged concentrically (see Fig. 3). It was difficult to avoid the conclusion that two different processes were simultaneously going on in the same testis. In none of the rabbits in this group did the testis become much enlarged; only one, however, was allowed to survive for more than 15 days. In later passages with this strain, a fairly intimate mixture of fibromatous and inflammatory lesions was frequently observed histologically. The results of passing the changed virus serially through rabbit testis are shown in Chart 1. Occasionally, as shown, virus was stored in glycerol in the cold before passages were continued. It will be seen how varied and unpredictable was the histological response in the rabbits' testes. Individual rabbits in the chart will be referred to presently.

Attempted Separation of IA and OA from Changed Virus

Acting on the supposition that this was a mixed strain containing both IA and OA viruses, I planned a number of experiments with a view to separating the two strains. First, virus was inoculated into the testis of a guinea pig, recovered 3 days later and passed again into rabbits; it was thought possible that one strain might survive better than the other in a foreign host. This idea was not supported, for the rabbit inoculated from the guinea pig testis showed lesions of mixed type. Secondly, rabbits were inoculated from changed virus diluted out to the limits of its infectivity. It was argued that if this virus really consisted of a mixture of two strains, it was unlikely that these were present in equal quantity; possibly, therefore, the inoculation of relatively few minimal infective doses might permit the recovery of one or other strain pure. In a first experiment, a rabbit (1-66, Chart 1) was inoculated intratesticularly with 0.001 cc. of "mixed" virus having a minimal infective dose for the skin of 0.0001 cc.; examination of its testis left no doubt that the strain was still mixed in character. A similar experiment was

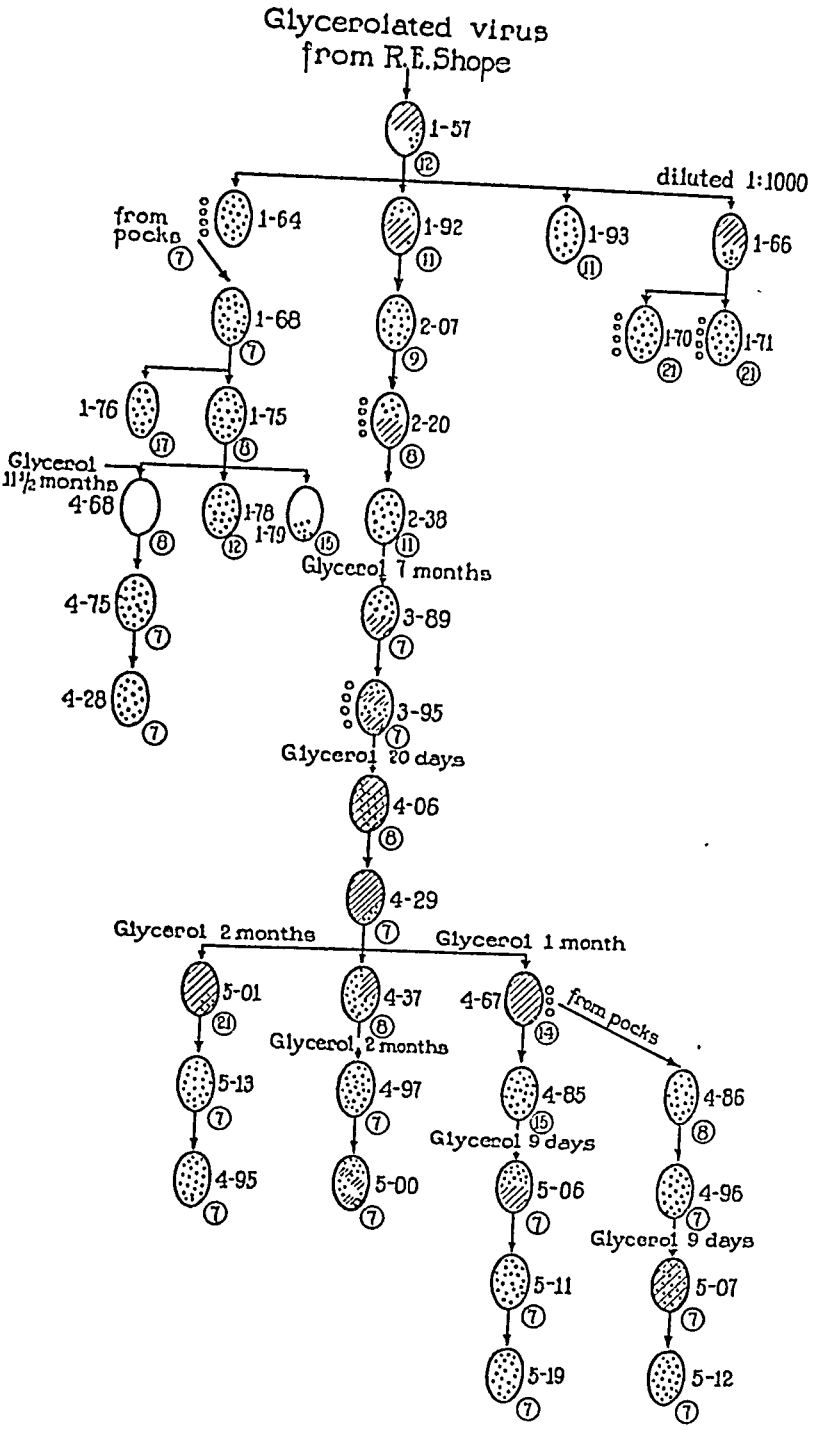

CHART 1. Diagram of passages of changed virus through rabbit testes.

A cross-hatched ellipse indicates a testis with fibromatous lesions. A dotted ellipse indicates a testis with inflammatory lesions. The nature of the lesions in other testes are suggested by various types of mixtures of cross-hatching and dotting. Figures at the sides of the ellipses are the serial numbers of the rabbits. The figures in circles indicate how many days passed between inoculation of the testis and killing the rabbit for passage or histological study. Small circles outside of some ellipses represent generalized pocks.

carried out with the B strain of virus which was at that time producing mixed lesions. A rabbit was inoculated into right and left testes with 0.01 cc. and 0.001 cc. of virus respectively. No lesions developed in the left testis, which received the smaller dose, nor in the testes of 2 rabbits inoculated in series therefrom. Small lesions appeared in the right testis, which thus presumably received less than 10 testis-infecting doses. Passages were made in series through 7 rabbits, and it was again clear that the virus was still mixed. I thus failed to recover a pure strain by this method, but my hope of succeeding perhaps rested on a fallacy; it is pure supposition that 1 minimal infective dose represents one virus particle; the small doses used for inoculation in these experiments may actually have contained several hundred virus bodies.

Next the attempt was made to recover pure OA virus by passing at long intervals. Shope recovered virus of the OA type from the testes of rabbits inoculated 21 and 35 days previously. The IA virus, however, proved in my hands difficult to isolate from testes injected a fortnight before. It was thought possible that the IA virus would in time disappear from a testis infected with the changed strain and passage was therefore made from a testis 3 weeks after inoculation (rabbit 5-01, Chart 1). The result was not what was expected, for the resulting lesions were predominantly inflammatory. The fourth method tried was the recovery of virus from one of the generalised pocks on the rabbit's skin. It was hoped to be able to pick out a pure "colony" conveniently "plated out" in this way. A rabbit (1-64, Chart 1) inoculated with changed virus developed only inflammatory lesions in the testis and also a generalised eruption on the skin. It has already been mentioned that in occasional rabbits inoculated with this strain only inflammatory lesions were seen, but that the mixed character of the strain was shown by the results of later passages. The rabbit was killed; several pocks were excised, ground with pyrex glass and saline and inoculated into the skin and testis of another rabbit (1-68). Typical lesions of IA type developed and from the infected testis virus was carried on in series. It was passed through 6 "generations" in all, involving 9 rabbits, and in none were any but inflammatory lesions seen. It seems fairly certain, therefore, that in this experiment a pure IA virus was recovered. At the time the experiment was carried out, generalisation had only been encountered in rabbits infected with IA virus. A year later, when both strains were liable to generalise, the experiment was repeated, but on this occasion the virus recovered from the excised pocks was still of mixed type (rabbits 4-67, 4-86, etc., Chart 1).

Experiment with B Strain of Virus

The B strain of virus, which has been briefly referred to before, was derived from a spontaneous growth in a different cottontail rabbit from that which originated the A strain. It has been studied by Shope and in England by my colleague, Dr. W. J. Purdy, and has regularly produced lesions of the fibromatous type. Dr. Purdy gave me two sealed ampoules of virus which had been dried; after receiving the virus from Shope, he had made only 2 rabbit passages before

drying this material. Rabbits inoculated with the dried virus from one ampoule showed only fibromatous lesions in the first 2 generations; thereafter the lesions were mixed in character, exactly like those of Shope's changed virus. Virus from the other ampoule also produced fibromatous reactions in the first 2 rabbits of the series; the 3rd rabbit showed mixed lesions; the testes of the 4th were not examined histologically. Those of the 5th to 10th inoculated in series showed an entirely inflammatory response like that of the IA virus, except that there was a small area with an atypical fibroblastic reaction in the rete testis of the 7th transfer rabbit.

It seems probable that the B strain of virus has undergone a change similar to that described for the A strain. It is unlikely that it could have been contaminated by my A strain for the following reasons. First, two separate ampoules dried in Purdy's laboratory at Mill Hill, where there had never been any A virus, yielded mixed and in one instance possibly pure inflammatory virus, on passage through rabbits at Hampstead. Secondly, I can confirm for the IA strain Shope's (1) finding that with the OA virus cross-infection of one domestic rabbit from another does not occur under laboratory conditions; I found no evidence of infection even when young animals were kept together in one cage, and when the intranasal route of infection was used.

It must be noted that in Purdy's hands the B strain did not lose its fibroma-producing properties after 21 passages; he always took care, however, to pass from rabbits in which the lesions appeared to be proliferative in type.

Artificial Mixtures of Strains

It was decided to study the results of mixing together pure OA and pure IA viruses in various proportions and passing from the mixtures in series through rabbit testes. The results of testing 12 such mixtures are shown in Table I. When the mixture contained a very large excess of OA virus, no signs of the presence of IA strain were evident after 4 passages (Experiment 4). Similarly with a tenfold excess of IA virus (Experiments 7 and 8), the OA virus was not apparent after 3 passages. The results of passing another mixture (Experiment 2) are shown in more detail in Chart 2. No differences were detected between the behaviour of this artificially mixed strain and the supposed natural mixture discussed earlier (see Chart 1); many inoculated testes when examined histologically showed lesions mixed in character. In

occasional rabbits the lesions were apparently almost entirely fibromatous or almost entirely inflammatory, but the testes were not examined in serial sections. It was very remarkable that on several occasions a rabbit inoculated with emulsions of an almost purely fibromatous testis itself developed almost wholly inflammatory lesions. Much less commonly the reverse occurred, "inflammatory" testes giving rise at the next passage to fibromata. As with the supposed natural mixture, there was no regular tendency for the lesions

TABLE I

Results of Inoculating Artificial Mixtures

Mixture	Minimal skin-infecting doses in mixture		Lesions in rabbit of			Total No. of passages
	IA	OA	1st passage	2nd passage	3rd passage	
1	(Virus diluted 1 in 2)	(Virus diluted 1 in 2)	OA	Mixed	Mostly IA	3
2	(Virus diluted 1 in 200)	(Virus diluted 1 in 2)	OA	OA	Mixed*	9
3	500	5000	OA			1
4	5	5000	OA	OA	OA†	4
5	500,000	5000	IA			1
6	5000	50	IA			1
7	500,000	50,000	IA	IA	IA	3
8	5000	500	IA	IA	IA	3
9	500,000	500,000	Mixed	Mixed	IA	3
10	5000	5000	IA	Mixed	IA	3
11	500,000	50,000	OA	Mixed	Mixed	3
12	50,000	500	Mixed	Mixed	Mixed	3

*Remained mixed in subsequent passages (*cf.* Chart 2).

†OA in 4th passage rabbit also.

in the series to become steadily more inflammatory or more fibromatous. One attempt was made to recover a pure virus from pocks on the skin of a rabbit receiving a mixture. This was carried out on a rabbit in Experiment 2 and the result is shown in Chart 2 (rabbits 1-40, 2-47); the virus recovered was, as in one of the experiments with the "natural mixture," of mixed character still.

Irregularity of Rabbit Response

The behaviour of the virus in the changed and artificially mixed series is in striking contrast to that of the pure OA and pure IA viruses.

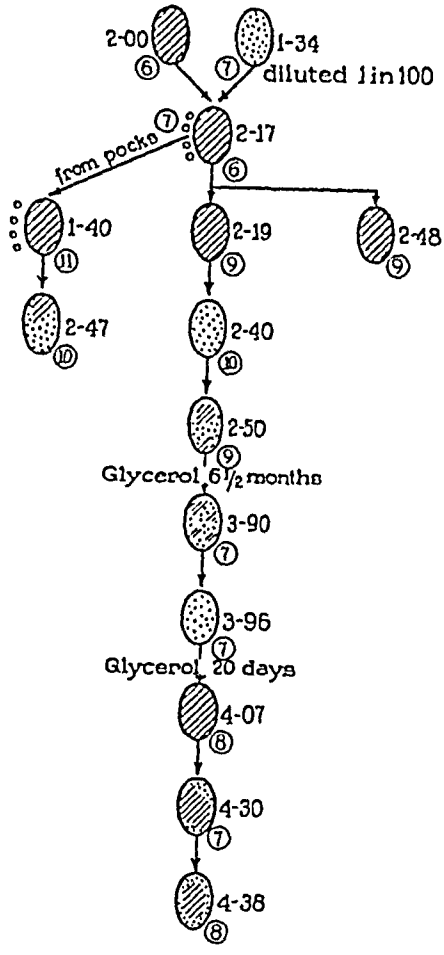

CHART 2. Diagram of passages of mixture of Virus OA and IA through rabbit testes. For explanation see Chart 1.

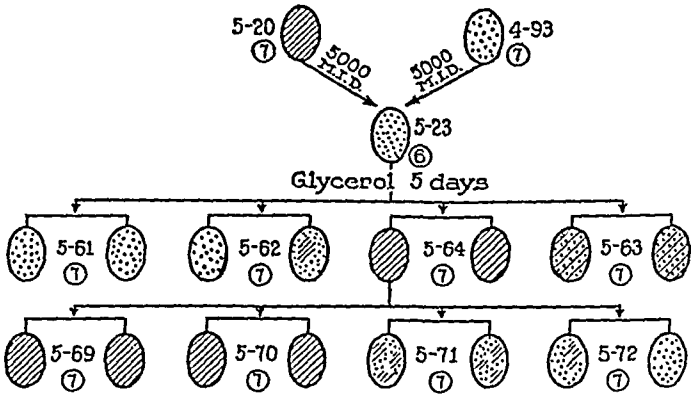

CHART 3. Diagram showing results of inoculating one virus mixture into different rabbits. For explanation see Chart 1. For most rabbits in this chart the histological responses in right and left testes are shown separately.

I have carried on the OA virus through 17 rabbits without detecting in it any tendency to change its character; the IA strain has similarly been passed serially through 32 rabbits and has at no time given rise to any fibromatous lesions. The recovery of pure IA virus from the changed virus and the exactly similar behaviour of the changed virus and artificial mixture make it seem almost certain that the changed virus is in fact a natural mixture. For a long time it seemed puzzling that the balance between the two constituents in the strain should have been maintained so evenly. The results of passing unequal mixtures (such as Nos. 4, 7 and 8 in Table I) suggest that if one strain came temporarily to preponderate, it might soon supplant the other completely. Doubtless this can happen at times and such an occurrence would explain the recovery of a pure IA strain from the virus originally sent me by Shope and later from the B strain of virus. But it is possible that irregularity in the response of rabbits to the mixture may ordinarily act as a stabilising factor. In this connection the experiment whose results are shown in Chart 3 is particularly interesting. Rabbit 5-23 was inoculated with OA and IA virus in equal parts and developed almost wholly inflammatory lesions in the testis. A centrifuged emulsion of this testis produced in 1 rabbit predominantly OA lesions; 4 other rabbits injected with the same virus after it had undergone a short stay in glycerol showed respectively wholly inflammatory, almost wholly inflammatory, mixed and wholly fibromatous lesions. A further passage from the last rabbit gave rise to lesions which were again very varied in character as the chart shows. All the rabbits received similar material into each testis and it was striking that the lesions in the right and left testes of each of the 8 rabbits were of the same nature. Thus any differences must have been attributable to something inherent in the individual rabbits and not to accidental factors. It is further clear that they were not due to qualitative differences in response to a single stimulus, but rather to the fact that some rabbits favoured the fibromatous virus in the mixture, others the inflammatory one. This must be so since either virus will regularly give rise to its own characteristic histological response when introduced by itself.

No attempt has been made to analyse the differences in rabbits which led to this result. But if rabbits favouring the IA virus and those favouring the OA were fairly equally distributed amongst the

stock used for passage, there would clearly be a tendency, normally, for a mixed virus to retain its mixed characters.

SUMMARY

A strain of rabbit fibroma is described causing in inoculated animals acute inflammatory lesions very different from the fibroma-like growths induced by the original strain. The new inflammatory strain cross-immunised with the normal strain but not with various other viruses. Efforts at changing one strain into the other were unsuccessful. Another, referred to as the changed, strain produced lesions of mixed character, partly inflammatory, partly fibromatous; it continued to behave in this way through numerous passages. An artificial mixture of inflammatory and fibromatous viruses behaved in all respects like the changed strain. Discussion of the significance of the findings is reserved for a separate paper (3) where the facts can be considered in relation to those described by Shope (2).

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EXPLANATION OF PLATE 14

FIG. 1. Section of testis of rabbit inoculated 7 days previously with OA virus. Early proliferation of fibroblasts in the interstitial tissues is shown. × 96.

FIG. 2. Section of testis of rabbit inoculated 7 days previously with IA virus. Intense infiltration with lymphocytic and mononuclear cells is evident. × 96.

FIG. 3. Section of testis of rabbit inoculated 15 days previously with changed strain of virus. The lower part of the picture shows the fibromatous (OA) type of response, the upper and right hand part the inflammatory (IA) reaction. × 96.

A CHANGE IN RABBIT FIBROMA VIRUS SUGGESTING MUTATION

II. Behavior of the Variant Virus in Cottontail Rabbits

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Andrewes (1) has observed a change in the type of lesion produced by a strain of the rabbit fibroma virus. Instead of causing fibroma-like tumors at sites of inoculation as at first (2) it produced in his experience only acute inflammatory lesions. The characteristics of this "inflammatory" type of virus have been described in an accompanying paper (1). A similar but less complete change in the virus has been noted in this laboratory. It is the purpose of the present paper to describe the change briefly and to outline experiments in which attempts were made to cause the virus to revert to the original type.

A Change in the Type of Lesion Produced by the Fibroma Virus

The virus used was the one studied in the original experiments (2) and designated, for convenience, as Strain A to distinguish it from viruses since obtained from fibromata found on 4 other cottontail rabbits. Originally and for a number of serial passages in domestic rabbits this virus had produced, when injected intratesticularly, a marked proliferation of connective tissue throughout the testicle and epididymis. When administered subcutaneously it produced at the site of inoculation a large swelling which had the gross and microscopic appearance of a fibroma. During the early intensive work with the virus it was passed at frequent intervals with at most short periods of storage in glycerol, while later it was transferred less frequently and after longer storage in glycerol. At each serial passage the virus was inoculated both subcutaneously and intratesticularly

into domestic rabbits, but usually only the fibromatous testicles were saved as a source of virus for infecting rabbits of the subsequent passage. Fibromatous infiltration of the testicles and subcutaneous tissue at the point of inoculation was noted at each passage. If the virus used in a given passage had been stored unusually long in glycerol the subcutaneous fibroma induced was in some instances quite small. This was thought to be due to loss or inactivation of part of the virus by prolonged storage. Direct passage from such a fibroma again yielded a typical large growth on subcutaneous inoculation.

No growth resulted at the site of subcutaneous injection in the rabbit of the 18th serial passage, but the 19th animal, injected with testicle from the 18th, developed a fairly large subcutaneous fibroma. Since then, in 12 further passages, the virus has failed to induce subcutaneous fibromata apparent in the gross. Andrewes had been supplied with virus from the 18th passage rabbit. It was thus evident that, at approximately the time that Andrewes noted that the fibroma virus no longer produced fibromatous lesions, a change had also occurred in the virus in this laboratory.

In addition to losing its ability to produce subcutaneous fibromata, my virus no longer caused the same extensive fibromatous proliferation in the testicles as formerly. The inoculated testicles, however, became firm, were swollen and inflamed, and scrotal edema was encountered quite frequently. Histologically, the picture seen in the testicle was now quite different from that originally produced by the virus. Instead of an extensive infiltration with young connective tissue cells, the spaces about the seminiferous tubules were densely packed with lymphocytes and large mononuclear cells. There was an accompanying atrophy of the tubule cells. As a rule only small and isolated islands of fibroblasts were to be seen, but in some passages the reaction was predominantly fibromatous. The subcutaneous tissue when inoculated with the virus also showed accumulations of lymphocytes and large mononuclear cells. The fibroblast-like cells so prominent formerly were now almost completely lacking. Virus administered intracutaneously produced raised, reddened, glistening papules which were similar histologically to the subcutaneous lesions. The change noted in the virus in this laboratory was thus apparently of the same general nature as that observed by Andrewes. It differed, however, in degree, for while Andrewes' virus produced only an inflammatory reaction when injected intratesticularly, the "changed virus" of this laboratory produced, in addition, a varying but usually scant connective tissue proliferation. Small satellite nodules were observed occasionally close to the inflammatory reaction at the site of subcutaneous injection or about the papules

arising where the virus was inoculated into the skin. The pock-like lesions described by Andrewes have not, however, been encountered.

Experiments with the Original Fibroma Virus

After several rapid serial transfers of the virus beyond the 19th passage had failed to restore its ability to produce fibromata when administered subcutaneously, the question arose as to whether the apparent change in the type of lesion produced was due to an alteration in the virus or to some variation in the host. So far as could be observed, the rabbits, now developing but scanty inflammatory reactions following subcutaneous inoculation with the virus, were similar in all respects to those earlier ones which had developed large subcutaneous fibromata. In order to settle the question, a second series of experiments was begun. The original cottontail rabbit fibroma which had furnished the virus under study had been in glycerol in the refrigerator for 18 months by now. A portion was ground, suspended in saline in the usual way, and used to inoculate a rabbit subcutaneously and intratesticularly. After an unusually long incubation period fibromatous nodules developed in the testicles. Passage from these yielded typical subcutaneous and testicular fibromata similar in all respects to those seen prior to the 18th serial transfer of the virus in the first series of experiments and described in an earlier paper (2). At this writing the virus in the second series of experiments has reached its 12th passage in domestic rabbits and it still induces large fibromata when injected subcutaneously, while changed virus of the first series of experiments, carried in parallel, has continued to produce only a scant inflammatory reaction when similarly administered. It thus appears that the change in the type of lesion produced was due to an alteration in the fibroma virus occurring during its 17th or 18th serial passage and not to any variation in the host.

Attempts to Restore Fibroma-Producing Properties to the Changed Virus

Since the natural host of the fibroma virus is the cottontail rabbit (genus *Sylvilagus*) (2) it seemed possible that its prolonged passage through a foreign host, the domestic rabbit (genus *Oryctolagus*), might be responsible for its modification. Instances in which viruses have been modified by transfer through unnatural hosts are well known. The question arose as to whether the change, in the case of the fibroma virus, was an irreversible one. Would passage of the changed

virus through cottontail rabbits restore its ability to induce fibromata when administered subcutaneously? To answer this question, virus of the 21st, 25th, and 31st serial passages was transferred intratesticularly to cottontail rabbits. The 21st passage virus was transferred through only 1 cottontail rabbit while the 25th and 31st passage viruses were submitted to 2 serial cottontail rabbit transfers. They were then carried back to domestic rabbits. In the first experiment the virus, after a single cottontail rabbit passage, induced large fibromata at the subcutaneous sites of inoculation in domestic rabbits of the first 2 serial passages. The inoculated testicles were diffusely infiltrated with fibroblast-like cells and the histological picture was identical in all appearances with that observed in rabbits infected with the original fibroma virus (2). In the 3rd domestic rabbit passage, however, the lesions both in the testicles and subcutaneous tissue were again composed largely of lymphocytes and large mononuclear cells with scattered islands of fibroblasts, indicating a reversion to the changed virus type of reaction. The other two experiments, in which the changed virus was submitted to two serial transfers through cottontail rabbits, yielded similar results. In one of these experiments the virus was transferred only once in domestic rabbits and yielded lesions at all sites of inoculation which histologically appeared to be purely fibromatous. In the remaining experiment the domestic rabbit infected with virus from the 2nd passage cottontail rabbit developed typical fibromatous lesions wherever inoculated, but in the 2nd domestic rabbit passage a reversion to the inflammatory type of reaction was observed. The cellular reaction in the testicles of all cottontail rabbits used in these experiments was composed predominantly of lymphocytes with scattered islands of fibroblast-like cells.

These experiments demonstrated that the change in the fibroma virus observed in this laboratory was at least partially reversible and that the mere passage of the virus back through cottontail rabbits was sufficient to restore temporarily its fibroma-producing properties.

Attempts to Restore Fibroma-Producing Properties to Andrewes' Inflammatory Virus

In June of 1934, Andrewes sent me his inflammatory strain of the fibroma virus (designated IA in the preceding paper (1)). It has been passed serially in this laboratory 11 times through domestic rabbits and has induced only inflammatory lesions when injected intracutaneously or intratesticularly. Unlike my own changed virus, Andrewes' virus gives rise to no fibroblast-like cells in the inoculated testicle; the cellular reaction is limited to lymphocytes, large mononuclear cells, and occasionally a few leucocytes. It is thus even more altered from its original character than is my changed virus.

Since its receipt, Andrewes' virus has been submitted to 5 transfers through each of 2 series of cottontail rabbits, the animals of each passage being inoculated both subcutaneously and intratesticularly with testicle suspension prepared from the preceding passage. Subcutaneous injections caused no reaction in most instances but occasionally a scant thickening was observed. Histologically, the reaction was composed entirely of lymphocytes and large mononuclear cells. The inoculated testicles showed evidence of infection at each passage. After an incubation period of from 5 to 8 days they felt abnormally firm, but did not increase in size as did testicles of cottontail rabbits inoculated with the original fibroma virus. On the contrary, where only one testicle was inoculated, it tended to be smaller than the uninoculated testicle, when the animal was autopsied on from the 12th to 15th day after infection. At autopsy the inoculated testicle appeared to be acutely inflamed. It was usually a mottled purplish red, and blood vessels in the tunica albuginea were tortuous and widely dilated. On cut section considerable serosanguineous fluid exuded, and the testicular tissue itself appeared soft and dark red in color. This picture was in marked contrast to that exhibited by testicles of cottontail rabbits infected with the original fibroma virus in which the cut surface was firm, white or pinkish white, and fibroma-like in appearance.

Histologically the testicles of cottontail rabbits infected with Andrewes' inflammatory virus resembled those of similarly infected domestic rabbits. The spaces about the seminiferous tubules were densely packed with lymphocytes and mononuclear cells, the tubule cells were in some areas necrotic, and occasional foci of leucocytic infiltration were encountered. No fibroblast-like cells, so common in testicles infected with the original fibroma virus, were to be seen.

No change in the type of lesion produced by the virus of either series of cottontail rabbit passages has been observed. Testicular lesions have been persistently inflammatory in character. 5th passage virus from both series, as well as that from earlier passages, has been transferred subcutaneously, intracutaneously, and intratesticularly in domestic rabbits, and in every instance the lesions have been inflammatory in character. The virus has failed to recover any of its ability to produce fibromata in either cottontail or domestic rabbits. The inflammatory virus has thus differed from my own changed virus in that cottontail rabbit passage has not re-endowed it with the property of inducing connective tissue hyperplasia at sites of inoculation in domestic rabbits. This difference in the two viruses will be discussed later (3).

The Identity of Inflammatory Virus and Fibroma Virus

Although the types of lesion produced in rabbits by the inflammatory and the original fibroma virus are distinctly different, cross-

immunity and cross-neutralization experiments described in the preceding paper (1) have indicated that the two viruses are immunologically identical. In this laboratory both cottontail and domestic rabbits have been tested for susceptibility to the original fibroma virus following recovery from infection with the inflammatory virus, and all were found to be completely resistant.

In earlier experiments (4) it was observed that domestic rabbits, which had recovered from infection with the fibroma virus, were usually resistant to the highly fatal *Virus myxomatosum*. An attempt was made to determine whether the inflammatory virus had the same effect. 26 days after infection with inflammatory virus, 6 domestic rabbits were inoculated subcutaneously and intratesticularly with *Virus myxomatosum*. 2 normal control animals infected in the same way died of typical infectious myxoma after 8 and 10 days. None of the 6 rabbits previously infected with inflammatory virus succumbed or showed any symptoms of generalized myxoma. 5 developed localized myxomatous lesions where inoculated, and 1 remained completely negative. This experiment indicated that the inflammatory virus bore a relationship to *Virus myxomatosum* similar to that shown by the original fibroma virus.

SUMMARY

A change in the type of lesion produced by the fibroma virus has been observed. At about its 18th serial transfer in domestic rabbits the virus lost its ability to induce fibromas when administered subcutaneously. Instead, lesions developing in either the subcutaneous tissue or in the testicle were predominantly inflammatory in character and partly composed of lymphocytes and large mononuclear cells in place of fibroblast-like cells. Andrewes, working with the same virus in England, has observed a somewhat similar change. Passage of my changed virus through cottontail rabbits results in a transient recovery of the capacity to produce fibromata, while similar passage of Andrewes' inflammatory virus is without effect.

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A CHANGE IN RABBIT FIBROMA VIRUS SUGGESTING MUTATION

III. Interpretation of Findings

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In the preceding two papers (1, 2) we have described aberrant strains of rabbit fibroma virus, a virus which normally gives rise to fibroma-like growths at the site of inoculation in cottontail or domestic rabbits (3). Those chiefly studied were derived from a growth in one cottontail rabbit (A); the strain in its original form is designated "original fibroma virus," fibromatous virus or OA strain. One of the atypical strains, first studied in England and designated as inflammatory virus or IA, produced only necrotic and inflammatory reactions on injection. The second was observed in America and called "changed virus;" the lesions produced by this in part resembled those of the original, in part those of the inflammatory strain. Similar changes were seen in the lesions produced by a virus originating from another cottontail growth (B strain), but they have not been as carefully studied.

Nature of the Changed Virus

Histologically, rabbit testes infected by the changed virus showed areas in which proliferation of fibroblast-like cells dominated the picture, together with areas in which there was an acute inflammatory reaction with much lymphocytic and mononuclear infiltration. Lesions of the two types were at times seen side by side, at times more intimately mixed.

These appearances suggested that the changed virus represented a

mixture of original and inflammatory virus. This explanation is rendered highly probable by consideration of the following facts.

1. Pure IA virus was isolated from changed virus on one occasion by excising and subinoculating from isolated pocks on the skin of a rabbit infected with the changed strain. Attempts to obtain pure OA virus, and other attempts at recovering pure IA virus, were unsuccessful.

2. The behaviour of the changed virus was exactly duplicated by mixing together OA and IA virus and making passages from the mixture. In both series, the two strains were carried along side by side, neither supplanting the other, and in both the histological picture was variable, commonly of mixed character.

3. Passage of changed virus through cottontails appeared to favour a fibromatous component, for after cottontail passage the lesions became predominantly of fibromatous type. The inflammatory element was not, however, eliminated, for on transfer to domestic rabbits the virus after only a few passages once more gave rise to lesions of mixed character.

Nature and Origin of "Inflammatory" Virus

The occurrence of the IA virus, provoking only inflammatory lesions, may be explained in one of several ways. First, it might be another virus which became mixed with the original strain; this second virus might be of domestic rabbit or of cottontail origin. Against this view are so many facts that it can probably be ruled out. (*a*) No virus answering to the description of the IA strain has previously been described. (*b*) Not only is there cross-immunity between the OA and IA strains, but no quantitative differences between immune sera prepared against the two can be demonstrated. (*c*) The two strains resemble each other in the frequent production of generalised pocks on the skin 6 to 8 days after inoculation of a rabbit. There is further the unexplained fact that with both strains this generalisation has been noted in England, but not in America. (*d*) Both strains occasionally produce cytoplasmic inclusion bodies in the epithelial cells of the skin of the domestic rabbit. These inclusions are like those which the OA strain produces in the cottontail. (*e*) Both strains will infect cottontail and domestic rabbits but so far as is yet

known, no other species of laboratory animal. (*f*) If a contamination of the OA strain with another virus occurred, a similar contamination of the B strain must also have taken place.

Secondly, it is possible also that the original cottontail rabbit material, obtained from the first naturally occurring case of fibromatosis, contained the two types of virus. This is unlikely because the virus bred true and produced characteristic subcutaneous fibromata for 17 serial passages in domestic rabbits. Not until the 18th transfer was there definite evidence that a change in the virus had taken place and all facts pointed to its being of sudden occurrence.

Thirdly, the inflammatory strain might be the result of gradual adaptation of the fibroma virus to existence in an abnormal host, the domestic rabbit. It is, however, difficult to believe that one strain of virus from the cottontail, when passaged in domestic rabbits in several series, should in one series retain its original character essentially unaltered and in another should change as a result of gradual adaptation to its new environment. Indeed, there is no evidence of gradualness; once the original virus had rather suddenly turned into changed virus, no further progressive modification in its properties occurred.

Fourthly, the inflammatory virus might be a variant or "mutant" of the original strain. The conditions suitable for such a mutation remain unknown, but if it were a rare event, it would not be surprising to find evidence of it in one series of passages through rabbits and not in another. Since the cottontail has been shown to favour the original as against the inflammatory strain, a mutant of the IA type occurring in this, its natural host, would probably be suppressed. Some tame rabbits have been shown to favour the development of the inflammatory virus and some the original strain; the fate of a mutant occurring in the tissues of the domestic rabbit would thus depend upon the reaction of the individual rabbits it encountered on its first occurrence and in the course of subsequent passages. The isolation of pure IA virus from the material first sent to England may have depended upon some differential effect of the conditions of the journey on the two strains in a mixture, or possibly on the chance that the virus encountered in England a succession of rabbits favouring the variant race.

Mutation amongst Viruses

We have used the term mutation to imply a variation which is abrupt, discontinuous and inherited. We are aware that some writers restrict the term to variation in higher animals and plants in which the variation is associated with certain changes in chromosomes. A phenomenon corresponding to mutation doubtless occurs in living things of simpler organization, and students of bacteria and viruses are left, if they agree to this restriction, without a word with which to express their meaning. The matter is, however, complex and we do not wish a discussion on terminology to obscure our main point.

We believe that the facts recorded by us are best explained on the hypothesis that the inflammatory virus is a mutant of the original strain. It would be important to prove conclusively the occurrence of an abrupt change in a virus such as this. All virus workers are familiar with changes in the properties of a virus such as increase or decrease in virulence or adaptation to a strange host. It is not clear whether this is usually thought of as a gradual change, or not; in most instances it would not be easy to analyse the phenomenon experimentally. The fibroma virus affords an exceptional opportunity for studying the nature of the change, for two reasons; first, the change occurs in a single animal species, and, secondly, there is a striking qualitative and not merely quantitative difference between the reactions produced by the two strains of virus. The tentative conclusion that an abrupt, discontinuous change is responsible makes it worth considering whether such an explanation could not cover many known facts about virus diseases. Thus the highly infectious rabbit pox described by Greene (4) might very well be due to a mutant strain of vaccinia of rare occurrence. Other, less dramatic, changes in viruses might be associated with the gradual supplanting of the original by the variant form, though this variant might from the first have differed widely from its parent.

The matter has come to the fore lately in several different fields of virus work. Abrupt changes in the character of the mottling symptoms produced by viruses have been noted for instance in the mosaic diseases of plants. McKinney (5, 6) and Jensen (7) were able to isolate viruses producing yellow mosaics from plants infected with tobacco mosaic virus. McKinney stated that the association of yellow mosaic virus with ordinary tobacco mosaic might not constitute

a contamination in the usual sense and suggested that the yellow mosaic virus might have arisen as the result of a mutation. Jensen proved that the viruses of yellow mosaics, many of which differed from each other, arose during multiplication of tobacco mosaic virus in infected plants. His carefully controlled experiments indicated that the yellow mosaic viruses originated from the tobacco mosaic virus and were not contaminations or viruses accidentally transmitted from some other plant. Price (8) found that tobacco plants infected with cucumber mosaic virus frequently developed bright yellow spots from which different but closely related yellow mosaic and necrotic type viruses could be isolated. He considered that his experiments indicated that strains of cucumber mosaic virus arose by mutation or a similar process in tobacco plants having the cucumber mosaic disease.

Asheshov and his coworkers (9) described "dissociation phenomena" amongst cholera bacteriophages which may be of the same nature.

Daubney (10) has used the term "mutation" to describe antigenic changes amongst strains of foot and mouth disease in East Africa. Incidentally if a virus should "sport" antigenically as he describes and at the same time change so as to produce an unwonted type of reaction, as recorded in our papers, it would probably be thought to represent an entirely "new" virus. Findlay and Clarke (11) as a result of their studies on the reconversion of neurotropic yellow fever virus to the viscerotropic strain, are led to discuss this question, but they favour the view that there are intergrades between two extreme types of virus. They admit, however, that they cannot exclude the possibility that there are two distinct types of virus particles with different tissue affinities, present according to circumstances in different proportions.

The Nature of the Difference between the Fibromatous and Inflammatory Strains

The observations we have recorded have an important bearing on quite a different problem. Different viruses induce in the cells they attack either necrosis or proliferation or both. The proliferation is most in evidence in the viruses causing tumours such as the fowl sarcomata and the rabbit papilloma. The fibroma virus is of special interest in that different strains of the same virus characteristically cause reactions of the two different types. There seems no doubt that the original virus acts by stimulating fibroblasts or their precursors to unwonted proliferation. It may be supposed, on the other hand, that the IA virus kills the cells it attacks, and that the inflammatory reaction is secondary to this cell-death and analogous to that seen in many other virus infections; it has been mentioned that in the early lesions in testes infected with inflammatory virus, numerous necrotic

cells are visible in the interstitial tissues. On this hypothesis, the OA virus attacks the cells less vigorously, causing them to proliferate but not killing them for some weeks, while the IA strain attacks with greater virulence or is met with a more violent response, so that rapid cell death results. An attempt was made to test this hypothesis by studying the effect of the two strains on cells growing in tissue cultures of rabbit testis (12). Unfortunately, only the inflammatory strain was successfully grown *in vitro* and even then no changes in the tissues which could be regarded as specific were produced.

SUMMARY AND CONCLUSIONS

A strain of rabbit fibroma virus (changed virus) producing partly fibromatous, partly inflammatory, lesions is believed to represent a mixture of the original virus with a strain (inflammatory virus) causing only necrotic and inflammatory lesions. The inflammatory virus does not represent a contamination from without, but probably arose as a mutant from the original strain. The occurrence of mutation amongst viruses and the propriety of using the word in this field are briefly discussed. Consideration is also given to the nature of the change in the virus which leads to a tissue reaction so widely different from that produced by the original strain.

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ON THE GROUP SPECIFIC A SUBSTANCE IN HORSE SALIVA. II*

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In the present paper, additional data will be given on the separation and properties of the substance contained in horse saliva that reacts with group specific immune sera produced by injection of human blood of group A.

The results gathered so far in regard to the substances characterizing the human blood groups appear to prove that their specificity is due to carbohydrate groupings, and thus relate haptens of animal origin to those existing in bacteria. The first experimental evidence along this line was obtained in the examination of the so called Forssman substance which is serologically allied to the group A substance (2). Thus, from horse organs in which Forssman antigen is present, highly active preparations were separated that on hydrolysis yielded fatty acids and a considerably higher percentage of sugar than the known lipoids (3).

The next significant information was gained when the Forssman substances as found in certain bacteria were recovered in polysaccharide preparations and were shown to be intimately connected and probably identical with the specific precipitable substance (4–7). Then there followed studies of Schiff and Brahn (8, 9) on the A substance of human origin and in commercial pepsin which led to carbohydrate preparations with about 5 per cent nitrogen, possibly in an amino sugar, and containing galactose.

The group substances in red blood cells which can be extracted by alcohol represent, according to Schiff, probably a combination of a water soluble form with lipoids, as may be assumed also for the Forssman hapten mentioned above and as has been fairly well demonstrated in the case of several bacterial substances.

Freudenberg and his coworkers (10–12) in the study of the group A substance in human urine obtained it in a polysaccharide preparation containing about 5 per cent N and 9–10 per cent N-acetyl. After hydrolysis 45–50 per cent reducing sugar was found, and galactose and glucosamine were identified. An active substance of similar elementary composition and properties, yielding 48.5 per cent sugar on hydrolysis, was prepared by the writer from horse saliva (1).

* First communication (1).

Additional evidence as regards the nature of the specific substance was afforded by enzyme and serological reactions. Thus it was shown that the activity of A haptens is destroyed by Morgan's *Myxobacterium* and *Saccharobacterium ovale* (13), microorganisms which have the characteristic property of decomposing bacterial polysaccharides (14, 15); the specific substance is likewise split by snail enzymes (12). Further, Witebsky, Neter, and Sobotka (16) found that A immune sera react with the acetyl polysaccharide of pneumococci of Type I, a result stressed by the significant observation of Freudenberg and Eichel who reported that they were able to restore the activity of the substance by reacetylation after it had been inactivated by treatment with alkali. Finally papers by Jorpes (17) are to be mentioned in which he suggests the protein nature of the substance from human urine which neutralizes anti-A agglutinins. Possibly his observations could be explained on the assumption that the A hapten occurs in a combination with protein of higher anti-agglutinating activity as compared with the free hapten.

Notwithstanding the importance of the results already achieved it does not seem superfluous to continue these chemical investigations. It is not certain whether the substances prepared represent approximately pure chemical individuals, and, although acetyl groups seem to play an important rôle, the structure essential for the specificity is not yet defined. This is emphasized by the chemical similarity in the substances which were obtained from urine of individuals belonging to groups A, B, or O (11). Furthermore, the examination of A haptens from various sources is of interest because as in the case of Forssman haptens, it is possible and indeed is indicated by experimental results that the sundry substances reacting with A antibodies are not identical.

EXPERIMENTAL

Highly active preparations, similar in composition, of the A substance in horse saliva were separated in several ways, *e.g.* preliminary purification could be effected by precipitating the active substance along with mucin with acetic acid, and removing the mucin through adsorption with Fuller's earth, or by removing proteins from the saliva by means of charcoal and kaolin. The following method gave satisfactory results.

Saliva was obtained from a horse salivated by injection of 100 mg. of arecoline hydrobromide in four portions at intervals of 20 minutes. 1 liter portions of saliva were evaporated to dryness on the steam bath, the reaction becoming quite alkaline. The dry residue was taken up in 50 cc. of water; a considerable amount

of insoluble material, after soaking for about 15 minutes, was removed by centrifuging. The supernatant fluid gave a strongly positive picric acid test. After neutralization with 50 per cent acetic acid the solution (40 cc.) was acidified with 6 cc. of 10 per cent acetic acid and the heavy precipitate removed by centrifuging. The supernatant fluid was neutralized with sodium carbonate; it was clear and no longer gave a precipitate with picric acid. The active material was precipitated by 1.5 volumes of acetone. After standing overnight the precipitate was collected and dissolved in 5 cc. of water, and a slight sediment centrifuged off. The solution in 10 cc. portions was brought to a boil with 1 gm. of animal charcoal at a reaction slightly alkaline to phenolphthalein. This adsorption was repeated once, or again with a smaller quantity of charcoal if the xanthoprotein reaction was not yet negative. Addition of 4 volumes of alcohol and some solid sodium acetate caused the formation of a flocculent precipitate which was centrifuged down and redissolved in 2 cc. of water. The procedures following were carried out with 50 cc. of solution representing 50 liters of saliva. Addition of 6 volumes of glacial acetic acid yielded an inert sediment which was removed by spinning; on further addition of 14 volumes of glacial acetic acid the active material precipitated. It was washed with alcohol and dissolved in 20 cc. of water. A sediment formed on adding 100 cc. of glacial acetic acid was discarded, and the substance precipitated by 220 cc. of glacial acetic acid. The material was washed with alcohol, dried, and dissolved in 20 cc. of water to form a viscous solution which no longer gave a xanthoprotein reaction. The solution was diluted with 1.5 volumes of alcohol and 1 gm. of sodium acetate was added. A sediment was separated by spinning. When a total of 2.5 volumes of alcohol had been added a gummy, sticky precipitate formed which was centrifuged down, redissolved, and refractionated with alcohol and sodium acetate. The substance was dissolved in 20 cc. of water and hydrochloric acid was added to N/10 concentration. Addition of 3 volumes of alcohol gave a slight precipitate which was removed and the remainder of the substance was precipitated with an excess of alcohol. To get rid of the last traces of charcoal the solution of the substance in 30 cc. of water was centrifuged at 18,000 R.P.M. After dialysis in cellophane bags, the substance was precipitated by the addition of 4 volumes of alcohol and a small amount of concentrated hydrochloric acid. After washing with alcohol and ether a white powder was obtained, the yield amounting to about 5 mg. per liter of saliva.

The various steps during the separation of the substance were controlled serologically. The activity was tested by means of immune sera hemolytic for sheep blood, prepared by injection into rabbits of human red blood cells of group A. To 0.5 cc. of progressively doubled dilutions of the solutions was added 0.5 cc. of 1:10 normal guinea pig serum and 0.5 cc. of diluted lysin containing 2 hemolytic units, determined after 15 minutes incubation. After 1 hour at 37° C. 1 drop of 50 per cent blood was added. The highest dilution still showing complete inhibition of lysis was taken as the titer; the readings were made when a control tube showed complete hemolysis.

In addition determinations of the inhibitory effect of the substance on the isoagglutination of A blood were made by mixing 1 volume each of a solution of the substance and human serum of group B, and adding 1 volume of a 1 per cent suspension of washed A cells after ½ hour at room temperature; after a short time the tubes were centrifuged and readings were made.

The substance analyzed as follows (calculated for ash-free substance): C 44.56 per cent; H 6.91 per cent; N 7.08 per cent; total S 1.78 per cent; sulfate S 0.05 per cent; P 0.23 per cent; acetyl[1] 9.40, 9.47 per cent; ash 1.20 per cent; reducing sugar after acid hydrolysis, calculated as glucose, 56.2, 57.6, 58.2 per cent.

The rotation in a 2.5 per cent aqueous solution at 30°C. was $[\alpha]_D$ = +10.0°. Another preparation under the same conditions gave a specific rotation of +10.8°.

The substance in 1 per cent solution gave negative reactions with picric acid, lead acetate, basic lead acetate, trichloroacetic acid, sulfosalicylic acid, tannic acid, ammonium sulfate, safranine, mercuric chloride, hydroferrocyanic acid, uranyl nitrate, and picrolonic acid. It gave a weak biuret reaction, negative or very faint xanthoprotein test, negative Millon test and ninhydrin reaction, a faint Sakaguchi test (18), and no coupling with diazonium solutions. There was no formation of lead sulfide on heating with lead acetate and alkali. A strongly acidified solution gave a heavy precipitate with phosphotungstic acid.

Fehling's solution was not reduced before hydrolysis. The Molisch reaction was intense. A sample after acid hydrolysis gave a strongly positive test for hexosamine according to Elson and Morgan's technique (19). The non-hydrolyzed material gave no reaction for hexosamine. The test for galactose by the formation of mucic acid through oxidation with nitric acid yielded 12.8 mg. of an insoluble acid, m.p. 213°, from 100 mg. of substance (obtained by a method of preparation other than the one described in detail). Tollens' reaction (20) for uronic acids was negative (light green ether solution), in Bial's orcin test (21) an intense violet color developed.

In the tests for inhibition of hemolysis the preparation proved to be of high activity, a 1 per cent solution being about 500 times as active

[1] The micro acetyl determinations were made through the kindness of Dr. W. F. Goebel.

as the original saliva. By inhibition of isoagglutination about 1/1000 γ of the substance could be detected. Quantitative tests were also made with the technique of hemolysis inhibition used by Freudenberg and his coworkers (22) which yields considerably higher absolute titers than the method mentioned above. In this way 1/2000–1/4000 γ of the substance was detectable. Since this value is ten to twenty times greater than that given for the preparations from human urine, comparative tests were made with the saliva substance and samples of the substance from human urine,[2] and in these experiments[3] the difference in activity was confirmed but was found to be considerably greater, likewise in tests of the inhibiting action of the substances on the isoagglutination of A blood.

COMMENT

From the data presented it is seen that in chemical composition and properties the horse saliva preparation is essentially, at least, carbohydrate in nature and is very similar to the preparations separated by Freudenberg from human urine. Thus the substance gives negative reactions with almost all protein reagents, yields on hydrolysis a high percentage of reducing sugar, contains about 10 per cent acetyl, and gives positive reactions for glucosamine and galactose. There were, however, the following differences as compared with the urine substance: the negative Tollens' reaction for uronic acids, the failure to give a precipitate with basic lead acetate, and, notably, the considerably higher serological activity. To explain this discrepancy one could assume that the urine substance contains a large amount of serologically inactive polysaccharides—in tests with A immune sera —or else that each of the two materials contains a different substance, both of them reacting with A antibodies, but unlike in reactivity. Preparations, still under investigation, made from commercial pepsin were found to be not less active than the one obtained from horse saliva.

The substance described gave a weak biuret test; whether this is

[2] These preparations were obtained from Professor Freudenberg and Dr. Jorpes to whom the author is greatly indebted.

[3] These tests were in part conducted by Dr. Witebsky with the method of the Institut fur Experimentelle Krebsforschung at Heidelberg.

due to an impurity has still to be determined. As to the violet color in the orcin test it is doubtful whether it is connected with the active substance since with other preparations possessing high serological activity the color developed was distinctly less intense. This color reaction was shown also by the substance from urine, which likewise gave a precipitate with phosphotungstic acid in strongly acid solution.

The experiments were carried out with the technical assistance of Mr. Jack Black.

SUMMARY

A method is described for the purification of the A substance in horse saliva, and additional observations on the chemical properties of the preparation are reported. The preparation isolated was found to be highly active serologically and it appears to be polysaccharide in nature.

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PATHOGENESIS OF PNEUMOCOCCUS INFECTIONS IN MICE

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PLATES 15 AND 16

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Webster and Clow (1) were the first to note that frank pneumonia could be consistently produced in unprepared mice. In addition, a certain number of resistant mice infected intranasally developed a cervical adenitis which might be acute or chronic but which resulted finally, in nearly every case, in septicemia and death with or without pneumonia. This suggested that the pneumococci may enter the body through the mucosa of the nose, pass to the lymphatics and so into the blood, and that the pneumonia may be secondary to a bacteremia. In the resistant mice the organisms are held up in the node, multiply, and produce an adenitis. Such ideas formed the basis for the present inquiry into the pathology (2) and pathogenesis of pneumococcus infections in mice.

Even as soon as it was established that the pneumococcus is the chief cause of pneumonia in man, the controversy as to the pathogenesis of the lesion produced by this organism began. The literature is very extensive and, therefore, apart from papers dealing with general principles, only those reporting work on mice will be discussed here. Fraenkel (3), in his original paper on the pneumococcus, described pneumonia in one rabbit given pneumococci subcutaneously. It was the experience of the majority of workers in the next twenty years, however, that pneumonia could only be produced in animals by the introduction of the pneumococci into the upper respiratory tract; and, as a result, Wadsworth (4) in 1904 stated that intravenous and, indeed, all methods giving rise to a primary general infection, with a few uncorroborated exceptions, failed to give rise to pneumonia. A search of the literature from 1886 until the present time shows that pneumonia has been produced through a primary general infection in very few cases. Apart, indeed, from Fraenkel's one rabbit, only one other case, that of Schultz (5), carries any conviction. It should be stated, however, that while positive reports

are few, negative ones, those namely in which a primary general infection was induced and failed to give rise to any pneumonia, are also scanty. It is now recognized, moreover, that pneumonias in animals may be produced by the parenteral inoculation of microorganisms other than pneumococci, as witness the work of Sisson and Walker (6) and Webster (7).

Both as a result of the work discussed above and because it was felt that in man the infection certainly occurred through the respiratory tract, attention was paid to this portal of entry. The most recent method adopted has been the instillation of a culture of pneumococci into the nose by Neufeld and Tulczynska (8) and Webster and Clow (1).

Since the introduction of pneumococci into the respiratory tract produced pneumonia, where in the tract did the invasion of the microorganisms take place, and by what route did they reach the alveoli? It was known that the organisms could invade from the lower trachea and bronchi and produce pneumonia when introduced into the respiratory tract at this point, as witness the work of Lamar and Meltzer (9), Terrell, Robertson, and Coggeshall (10), Blake and Cecil (11, 12), and Kline and Winternitz (13, 14).

On the other hand, Neufeld and Tulczynska (8) believe that when mice are infected intranasally with pneumococci, infection may occur through the mucosa of the nose. They do not report the production of pneumonia, but Stillman and Branch (15) and Griffith (16) were able to produce pneumonia in previously prepared mice by spraying, while Webster and Clow (1) were the first to obtain definite pneumonia consistently in untreated mice by intranasal instillation of pneumonia. Stillman and Branch thought that in their mice the initial lesion was in the alveoli, but they noted cases in which there was only a serofibrinous pleurisy. In a later paper (17) they recognize the possibility that the infecting agent may reach the alveolar wall by the blood stream.

Clark (18) was able to show in rabbits that potassium ferrocyanide and iron ammonium citrate could pass within an hour through the mucosa of the nose entering into the perineural sheaths to pass to the brain, into the lymphatics to pass to the cervical nodes, and, to a lesser demonstrable extent, into the tissue spaces and blood vessels themselves. He noticed that passage occurred almost entirely through the olfactory as compared to the respiratory mucosa. There has been no other satisfactory study of the mechanism and rapidity of absorption through the mucosa of the nose and upper respiratory tract.

It has been shown repeatedly that as a result of exposing animals to a spray of infected culture, many organisms rapidly reach the lungs. Wherry and Butterfield (19) found *B. enteritidis* in the lungs of mice after 30 minutes' spraying. Stillman (20) found that the lungs of mice contained pneumococci after 10 to 15 minutes' spraying with pneumococci which persisted up to 3 hours. Lange and Keschichan (21) found pneumococci plentiful in the lungs of mice after 30 minutes' spraying, and Lange and Nowosselsky (22) found them in the lungs immediately after 15 and 30 minutes' spraying. There is also considerable evidence as to the rate of penetration of particles through the alveolar walls. Thus, recently Fang,

Field, and Drinker (23) showed that particles of nickel and magnesium silicate, injected into the trachea, reached the alveoli promptly and the bronchial nodes in less than an hour. Stillman (24) found pneumococci in the blood culture in two out of six alcoholized mice sprayed and killed at the end of an hour. Frequent positive blood cultures appeared at 6 hours or later. Normal unalcoholized mice did not show positive blood cultures. On the other hand, Lange and Nowosselsky (22) were unable to obtain positive blood cultures at any time between 1 and 48 hours after spraying mice with pneumococcus cultures. Neufeld and Tulczynska (8), Stillman and Branch (17), and also Robertson, Coggeshall, and Terrell (25) recognize the possibility of transient bacteremias that might escape notice in scattered blood cultures.

A further debated point has been the route by which the pneumococci, once within the body, reach the alveolar walls to produce the lesion. Of course, those who believe that infection occurs through the walls of the alveoli think that the organisms produce the first changes either before they pass into the alveolar walls or when once within the alveolar walls. If, however, the infection occurs at some other point in the respiratory tract, the organisms may reach the lungs either through the blood stream or the lymphatics. Owing to the very scanty evidence that pneumonia can be produced by direct infection of the blood stream, the first possibility has been largely discounted. The lymphatic route, on the other hand, has received support from Blake and Cecil (12) and Permar (26) who believe that invasion occurs in the main bronchi and that there is a retrograde spread of the infection back through the lymphatics to the alveolar walls. The studies of Cunningham (27), Kampmeier (28), and especially of Miller during many years (29) in several animals have made clear the direction of lymph flow in the lung.

The lymphatics may be divided into a superficial pleural plexus and a deep plexus along the blood vessels and bronchi which are connected by occasional channels. In the superficial plexus the flow is eventually into larger trunks which empty into nodes at the hilum. The flow in these larger trunks is directed by valves whose concavity faces to the hilum. In the deep plexus the lymphatic vessels run on the bronchi and blood vessels, communications between these various networks being frequent. Miller (29) has shown that the lymphatics end on the ductuli alveolares and that there are no lymphatics in the walls of the alveoli. The valves in this deep plexus are arranged so as to ensure that the lymphatic flow shall be towards the nodes at the hilum.

This disposition of the valves in the lymphatics would seem to exclude the possibility of reverse or to and fro flow in these channels. Even should a retrograde flow be possible, the infection could not reach the alveoli themselves without passage through the lymphatic walls.

Wadsworth (4) was the first to introduce the important concept of a balance between the virulence of the organism and the resistance of the host in the production of pneumonia. Griffith (16) also pointed out how dependent the production of an infection with the pneumococcus by inhalation was on the invasive

quality of the culture used as apart from the intraperitoneal virulence. Webster and Clow (1) point out that the response of mice to intranasal inoculation of pneumococci varies with the resistance of the host and the intranasal virulence of the strain of organisms. This concept of the balance between the various powers of the pneumococcus and the general resistance of the host is of the greatest importance in the production of pneumonia in mice, as will be pointed out.

TABLE I

	Minutes															Hours	
	1	2	5	7½	10	15	20	22½	30	40	45	50	60	80	90	100	2	3
Cervical nodes	0/4	0/4	0/4	0/4	0/5	0/2	0/3	—	0/3	0/3	0/2	0/3	0/4	0/1	0/2	0/1	0/2	0/2
Inguinal nodes	0/4	0/4	0/4	0/4	0/5	0/2	0/3	—	0/3	0/3	0/2	0/3	0/4	0/1	0/2	0/1	0/2	0/2
Trachea	3/3	1/3	2/3	1/3	2/3	1/3	—	0/3	1/3	—	2/3	—	2/3	—	3/3	—	0/3	0/3
Lung	0/7	4/7	4/7	1/7	1/8	3/5	2/3	1/4	5/8	1/3	1/5	2/3	3/8	1/1	0/5	1/1	0/6	1/6
Heart	0/7	2/7	0/7	0/7	1/8	0/5	1/3	0/4	3/8	0/3	0/5	2/3	1/8	0/1	1/5	0/1	1/6	0/6
Spleen	0/7	2/7	0/7	0/7	1/8	0/5	2/3	0/4	1/8	0/3	0/5	1/3	1/8	0/1	0/5	0/1	0/6	0/6
Kidney	0/7	2/7	0/7	0/7	1/8	0/5	1/3	0/4	1/8	1/3	0/5	2/3	1/8	0/1	0/5	0/1	0/6	0/6

Unselected albino mice killed by chloroform.

The figures shown represent the number of positive cultures/the number of cultures made.

TABLE II

Organ	Minutes			
	1	3	5	11
Trachea	9/11	10/12	8/12	7/12
Lung	3/11	2/12	6/12	2/12
Heart	3/11	2/12	6/12	2/12
Spleen	2/11	1/12	2/12	3/12
Invasion of blood (heart and spleen)	4/11	3/12	7/12	4/12

Swiss mice killed with clamp.

In summary, it can be said that three possible routes by which the pneumococci reach the alveolar walls to produce the pneumonic lesion have been investigated. These are: direct transport to the alveoli through the respiratory tract, a hematogenous infection, and a lymphatic infection. Of these, the second is generally thought not to have any importance. A certain amount of negative evidence has been brought forward to show that a blood stream infection cannot

produce pneumonia, but there is some slight evidence to suggest that it can. It has been shown that particles can be rapidly absorbed through the olfactory mucosa of the nose. The lymphatic spread is upheld chiefly by those who believe that the point of invasion is the walls of the large bronchi. The organisms, having passed the mucosa, are presumed to enter the lymphatics and pass in a retrograde direction to reach the alveolar walls. There is, however, some anatomical evidence which renders such a method of spread doubtful. Pneumonia can be produced when the pneumococci reach the alveoli themselves in any manner through the respiratory tract. Under such circumstances, the organisms, in common with other animate or inanimate particles, quickly penetrate the alveolar walls. Once inside the walls, the organisms spread either through the tissues, through the lymphatics, or through the blood vessels. Finally, there has developed the concept that in order to produce infection and pneumonia in the host there must exist a particular balance between the invasiveness and virulence of the organism on the one hand and the relative local and general susceptibility of the host on the other.

RESULTS

As a first step in the investigation of the pathogenesis, it was determined from which organs pneumococci, dropped into the nose, could first be cultured. The material and technique employed were those already outlined elsewhere (2).

Mice were inoculated intranasally with a heart's blood culture of Type III pneumococci, undiluted or diluted 1/100 in saline. In early experiments they were killed at stated intervals thereafter by chloroform anesthesia and later by clamping a heavy clamp across the neck as high up as possible. This clamp macerated the cervical cord and closed off the trachea, causing rapid death with no agonal gasping respiration. Cultures were taken, with sterile precautions, of cervical and inguinal nodes, heart's blood, trachea, periphery of the lower lobe of the lung, spleen, and kidney, which were minced with scissors and placed in pneumococcus broth. Each tissue, of course, contained blood, and if the blood were heavily infected, the results were equivocal. The results of all the experiments are given in Tables I and II.

Pneumococci in the Lower Respiratory Tract

It will be seen from Table I that the cervical nodes never gave positive cultures. It is possible that organisms may have been

present in the nodes, especially later, but in numbers too small to be demonstrated by the method adopted. On the other hand, the other organs showed positive cultures surprisingly early. This was especially true of the respiratory tract—the trachea and lung. Both of these gave positive cultures within the first minute or two, and continued to give frequent positive cultures during the first hour. Later, the organisms could not be demonstrated so readily.

That the positive cultures from the trachea are not due to the presence of organisms in the blood is shown by the greater frequency of the former than of the latter. Reference to Table III, the protocol

TABLE III

Mouse No.	Time	Trachea	Heart	Lung	Spleen
	min.				
1	1	++++	++++	++	0
2	1	++++	0	0	0
3	1	++++	+	0	++
4	3	+++	0	0	0
5	3	++++	0	++	0
6	3	++	0	0	0
7	5	+++	0	0	0
8	5	+++	+	0	0
9	5	+++	+	0	0
10	11	++	0	0	0
11	11	++++	+	++	+
12	11	++++	++++	0	0

of a single experiment, makes this point clearer. In considering the comparative number of organisms which could be demonstrated, it should be remembered that the amount of blood used in the heart's blood culture is at least 100 times as much as that included in the small piece of trachea taken for culture. That positive lung cultures are due to some extent to positive blood cultures is more likely. However, if reference be made to Table III it will be seen that the two organs do not necessarily show positive cultures at the same time, and this has been true in all of the experiments. A certain number of positive lung cultures therefore represent pneumococci in the alveoli as apart from the capillaries.

Pneumococci in the Blood

If reference be made again to Tables I and II, another fact is immediately obvious—namely, the surprisingly early positive cultures from the heart's blood, the spleen, and the kidney. The positive cultures from the latter organs represent, of course, blood stream infection. It will be noticed that organisms can be found in the blood as early as they appear in the lower respiratory tract, thus pointing to a rapid invasion of the tissues and blood stream by the pneumococcus. This observation was further investigated.

Mice were inoculated intranasally and blood cultures were taken at stated intervals from the tail. A sterile 1 cc. pipette with a fine internal bore at the tip was used for each culture. This pipette was held in a clamp with its tip downwards while the other end of the pipette was connected, by means of rubber tubing, with a 2 cc. pipette. When blood was to be taken, the tail was sterilized, the tip cut off and the first drop of blood wiped away with a sterile sponge. The tip with a fresh drop of blood was then brought close to the tip of the pipette, and blood flowed into the latter by capillary attraction. More blood could be obtained by withdrawing the plunger of the syringe. About 0.05 cc. was taken for each culture and was expelled into the tube containing 1 cc. of pneumococcus broth.

Experiments were conducted with five breeds of mice: resistant, susceptible and unselected albinos, white-face, and Swiss mice. Type III cultures were used in all tests because this type has proven itself most frequently intranasally virulent and because it is most easily recognizable in culture.

Table IV gives the results. The columns show the percentage number of mice with bacteremia at any time, and the percentage of deaths in any breed. The bacteremia is further analyzed into two parts: (1) the bacteremia during the first 10 minutes, and (2) the bacteremia from 24 hours to death. It will be noticed in the first place that 32.9 per cent of all mice tested gave positive blood cultures during the first 10 minutes following inoculation. This result had been foreshadowed by the results already expressed in Tables I and II, but the figure is nevertheless surprisingly high. When it is considered that the technique is by no means certain of demonstrating very small numbers of organisms, and that the single drop of blood taken might well not contain the one or two organisms which have penetrated the mucosa and reached the peripheral blood stream, it

will be realized that this figure represents only the lowest estimate of early bacteremias that occurs. It is possible that all of the mice have this early bacteremia but that the present technique is incapable of demonstrating it with greater frequency.

The analysis of the bacteremia shows, moreover, that while the correlation between the percentage mortality in the different breeds of mice and the percentage of late bacteremia is very close, there is no such correlation with the early bacteremias. Thus, the early bacteremia has little if any bearing on the ultimate outcome of the inoculation, whereas the later cultures represent a septicemia and indicate a fatal outcome. This lack of significance of the early bacteremia and

TABLE IV

Comparison of Bacteremia in Different Strains of Mice Following an Intranasal Instillation of Pneumococci, Type III

Strain of mice	No. of mice	Bacteremia any time	Bacteremia within 10 min.	Bacteremia 24 hrs. to death	Died
		per cent	*per cent*	*per cent*	*per cent*
White-face	25	88.2	48	83.3	84
Swiss	24	66.6	25	—	96
Bacteria resistant	25	52	28	38.5	36
Bacteria susceptible	25	64	32	50	44
Unselected	38	81.6	31.6	61.6	81.6
Total	137	71.5	32.9	59.4	69.4

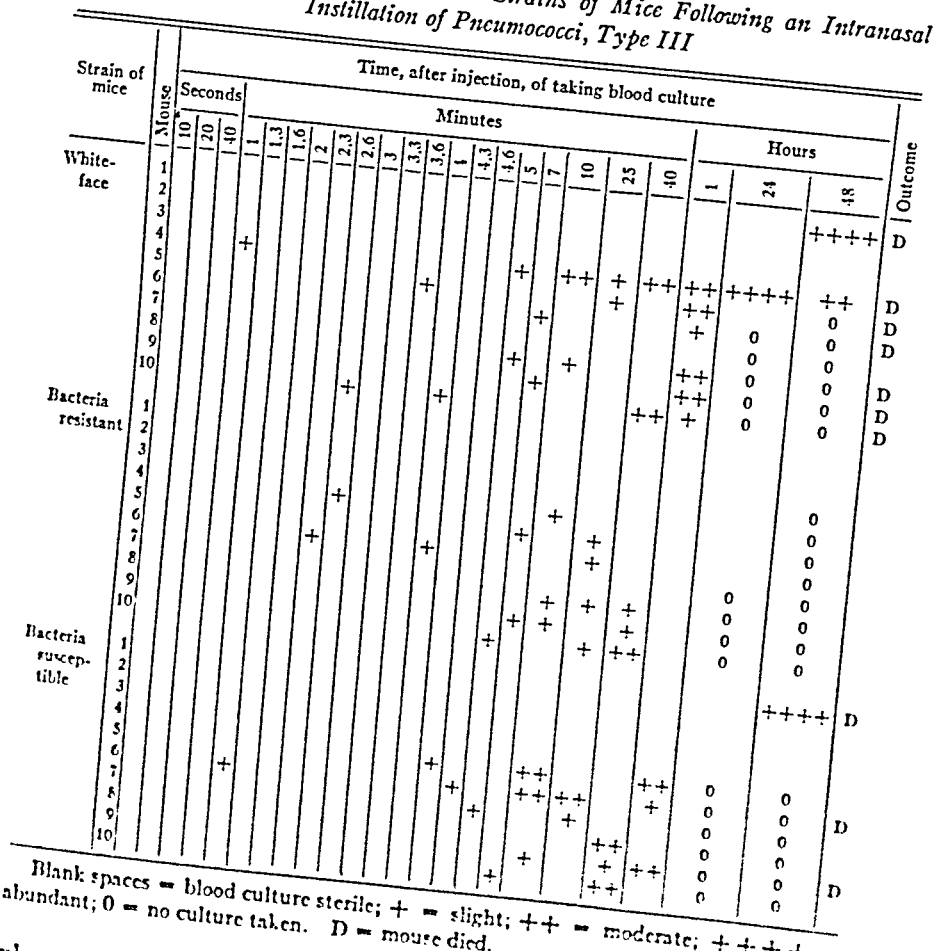
the serious import of the later septicemia are brought out by Table V, the protocol of one of the experiments. It is seen that the early bacteremia occurs with the same frequency in all breeds, for six white-face, six resistant, and five susceptibles give positive cultures during the first 10 minutes. This, however, has no relation to the mortality since seven white-face, no resistants, and three susceptibles died. On the other hand, the later invasion of the blood stream, in those cases in which it was tested, corresponds closely with the ultimate outcome.

Since bacteremia occurs so rapidly, there can be little doubt that the pneumococci are able to penetrate the mucosa of the respiratory tract with great rapidity and in this way to gain the tissues and the

blood stream. The speed with which such invasion occurs would seem to indicate that in this case, at least, the organisms penetrate the undamaged mucosa by themselves and not within the bodies of

TABLE V

Comparison of Bacteremia in Different Strains of Mice Following an Intranasal Instillation of Pneumococci, Type III



Blank spaces = blood culture sterile; + = slight; ++ = moderate; ++++ = abundant; 0 = no culture taken. D = mouse died.

phagocytic cells, although it is generally held that the majority of particles introduced into the alveoli are taken up by "dust" cells and enter the tissues inside these cells. There is no indication at the

moment, however, as to the exact manner in which this invasion takes place.

Point of Invasion

Of great importance in the consideration of the pathogenesis of pneumonia in mice is the question as to the point at which invasion actually occurs. It has been shown that, with the technique of intranasal inoculation adopted, the organisms reach both the lung and blood stream almost immediately. The question is, therefore, which tissues are first invaded—those of the lower respiratory tract or those of the nose with subsequent invasion of the lung. The work of Silvast (30) and others has already demonstrated that invasion of the tissues and lymphatics of the lung by passage through the alveolar walls was a matter merely of a few minutes. One cannot, however, ignore the possibility that some invasion occurs through the nasal mucosa. Neufeld and Tulczynska (8) believed that some infection took place here, and Clark (18) showed that the absorptive powers of the olfactory mucosa are very good. The occurrence of cervical adenitis while all the other peripheral nodes are unaffected, which we have described, points to an invasion at some point in the upper respiratory tract, as perhaps does also the very rapid appearance in the peripheral blood.

The chief reason perhaps for believing that invasion by the pneumococci of the upper respiratory tract, if it should occur at all, was unimportant in the pathogenesis of pneumonia was the belief that pneumonia could not be produced by any method causing a primary general infection, that is, intravenous or subcutaneous inoculation and the like.

Hematogenous Pneumonia

Wadsworth (4) had emphasized the importance of obtaining a balance between the virulence of the organisms and the resistance of the host. He pointed out that, unless this were done, pneumonic lesions probably would not be produced. It seems, however, that no investigator has ever applied this suggestion to experimental pneumococcus infection by any other route than that of the upper respiratory tract.

Work on the production of pneumonia by means of intranasal inoculation (1, 2) has shown that the careful choice of both strain of organism and breed of mice is essential if pneumonic lesions are to be obtained. If strains which have not been tested previously for their intranasal virulence be used indiscriminately as they reach the laboratory from the clinic, it will be found that, if Type III strains be omitted, the majority, no matter how high their intraperitoneal virulence may be, will fail to produce death or disease in the most susceptible breeds of mice on intranasal inoculation. The breed of mice also is an important factor (Table VI). This knowledge was applied to the study of hematogenous infections.

It was probable, on theoretical grounds, that if the virulence of the organisms was too high or the resistance of the host too low, death

TABLE VI

Comparison of Different Breeds of Mice Following Intranasal Instillation of 30,000 Pneumococci

Pneumococcus type	Mouse strain	No. tested	Pneumonia	No. dead	Per cent dead
III	Bacteria resistant	20	2	5	25
	Bacteria susceptible	20	8	13	65
XIX	Bacteria resistant	20	1	2	10
	Bacteria susceptible	20	8	11	55

would be so rapid that no lesions would have time to develop in the lung, whereas an organism of low virulence or a host of very high resistance might preclude the development of any lesion whatsoever and the mouse would survive. Preliminary experiments soon showed that such was indeed the case. Thus, all the mice, even the most susceptible breeds, would survive and remain well following intravenous doses of virulent strains of pneumococci when these doses were too small, or would survive and remain well following relatively large doses of pneumococci of low virulence. On the other hand, large doses of virulent strains in any breed of mice caused death so rapidly, *i.e.*, within 20 hours, that the lesions did not develop. Moreover, certain breeds of mice, especially the more resistant, were less prone to develop pneumonia under any circumstances. However, when the

dose and virulence of the organisms were so regulated to the relative resistance of the host that the latter survived about 2 days or longer and then died, it was found that pneumonia developed.

Technique

All intravenous inoculations were made into the tail vein, in 0.5 cc. amounts, through a 27 gauge ¾ inch needle.

Four strains of pneumococci were used. Of these, three, *i.e.* Type II, Type III, and Type XIX, were virulent intraperitoneally and intranasally, while the fourth, a Type I, was virulent only intraperitoneally. Resistant, susceptible, and, on one occasion, Swiss mice were used.

As far as concerns the lesions in the lungs, there was little difference to be noted between those produced by the four different types of pneumococci employed. Nor was there any essential difference in the breeds of mice. Indeed, the lesion appeared to be dependent directly on the length of survival time following inoculation and on that alone. The pulmonary changes will therefore be described as a group.

Macroscopically, consolidation was noted in twelve out of the 87 mice, and serofibrinous pleurisy seventeen times. Marked generalized congestion of the lungs was commonly found.

Microscopically, six of the 87 mice showed no lesions of any sort. Seventeen showed early and 55 more advanced lesions. These consisted of the collection of fluid, monocytes, and polymorphonuclear leucocytes in the interstitial tissue of the alveolar septae, resembling the early lesion described in Type III intranasal infections. This lesion developed and in the advanced stages the alveolar walls were many times their original thickness due to the infiltration of fluid and cells, many of the latter being polymorphonuclear leucocytes (Figs. 1 and 2). The advanced interstitial lesion was associated in many cases with an exudate of fluid and a few cells into the alveoli (Figs. 3 and 4). In no case did the number of leucocytes in the alveolar exudate become so great that the exudate was predominantely cellular. This fact was due presumably to the relatively short survival time which in no case exceeded 4 days, for the other lesions were indistinguishable from the first three stages of the pneumonia produced with Type III pneumococci introduced into the nose (2), and there is no reason to suppose they would not progress similarly. In the Type I intravenous infection, perivascular lymphatic dilatation was not observed but it was present with the three other types and the dilated lymphatics were filled with fluid and cells. The serofibrinous pleurisy found in about one-fifth of the mice was often extensive, the

pleura being covered with a thick layer of cells and fibrin and the pleural cavity filled with gelatinous or clear fluid.

With regard to the lesions in the other organs, it was found that they differed in infections due to different types of pneumococci and that there were also differences in the two breeds of mice. The cervical nodes were never affected, thus demonstrating that the lesions noted in the intranasally infected group were due to penetration of the organisms through the mucosa of the upper respiratory tract. The spleen showed necrosis of the follicles in both Type III, in which the susceptibles were most affected and the Swiss least, and in the Type XIX infection in which nine out of ten susceptibles were affected and only one resistant mouse. The lesion was usually so advanced that it could be recognized macroscopically. No necrosis of the follicles or other lesion was noted in the Type I and II infections. Necrosis of the liver was noted in all four types. In one, the Type II infection, the lesion was more pronounced in the susceptible than in the resistant mice. The kidneys were normal in Type III infections in complete accord with past experience (2, 31, 32); they showed acute diffuse nephritis infrequently, and lesions of the convoluted tubules rather more frequently, in Type I and Type II infections; and acute diffuse nephritis was present in nearly every case of Type XIX infection. No difference in the degree or type of change in the kidney was noted in the different breeds of mice.

Serofibrinous peritonitis was found in three out of the twenty mice inoculated with the Type I strain.

Pneumonia, therefore, can be produced in mice when the pneumococci are introduced by the intravenous route. At present, the only factors known to be important are the choosing of the strain of organism, the dose given, and the breed of mice, in such a way that death shall not occur too rapidly. If survival be a matter only of a few hours, pneumonia will not have time to develop. In the investigation described above, it was found that lesions very rarely occurred when the survival time was under 30 hours and, on the other hand, that almost all of the mice which failed to show any signs of localization in the lungs died in less than 20 hours. The pneumonia did not vary significantly with the various serological types of organisms used and was produced as readily with a strain of no intranasal virulence as with one of high intranasal virulence. In general, the picture resembled the first stages of that produced by a Type III strain when inoculated intranasally. The advanced stages of pneumonia with marked cellular exudate were very rarely seen, presumably because of the relatively short survival time.

DISCUSSION

It has been shown that pneumococci dropped into the uninjured noses of unprepared mice are able to produce pneumonia with a frequency depending on the virulence of the organism and the relative susceptibility of the host. The importance of the balance of both of these factors in experimental pneumonias has been long recognized (4), but it is only recently that the further important factor of the intranasal or respiratory tract virulence as compared with the intraperitoneal virulence has been recognized (1, 16). If Type III strains be omitted, it is found that the great majority of other pneumococcus strains, whatever their intraperitoneal virulence, lack the power to invade and cause disease or death when dropped on the uninjured mucosa. Such strains are useless for the production of pneumonia intranasally in mice and may explain many of the reported failures to produce pneumonia in mice not previously treated in some way or other.

The frequency with which pneumonia can be produced in susceptible breeds of mice with these pneumococcus strains of high intranasal virulence has made it possible to attempt an analysis of the pathogenesis of the infection as it occurs in mice, and particularly of the pneumonia.

It has been shown that, with the technique adopted, the organisms reach the lower respiratory tract and the alveoli almost immediately. Positive cultures can be obtained from the trachea and alveoli during the first minute. At the same time, the blood contains pneumococci which can be demonstrated in a third of all mice in the first 10 minutes and, indeed, in a few mice within the 1st and 2nd minutes after inoculation. It seems probable that the organisms which enter the blood stream invade at more than one point in the respiratory tract. The lesion of the cervical nodes, the extreme rapidity with which the blood becomes positive, and the fact that the absorptive capacity of the olfactory mucosa is high (18) all point to an invasion in the upper respiratory tract; the fact that the organisms rapidly reach the bronchi and alveoli, and previous work on the rapid passage of particles, fluids, and organisms from the alveoli into the tissues (30) point to invasion in the lower respiratory tract. No evidence has been forthcoming as to the method by which this invasion occurs, but its rapidity

would seem to exclude the possibility that the organisms are phagocytosed as they lie on the mucosal surface and enter the body inside these cells.

An analysis of the protocols and total figures of the blood cultures in mice receiving intranasal instillation brings out evidence on two points. In the first place, it is clear that the positive cultures during the first few minutes represent a bacteremia produced by the passage

TABLE VII

Dilution	Mouse No.	Minutes						Hours				Time of death
		3	6	8	11	29	47	1	2	4	6	
												hrs.
10^{-4} 20 organisms	1	0	0	0	0	+	++	0	+	++++	++++	27
	2	0	0	0	+	0	0	0	+++	++++	++++	16
	3	0	0	0	++	++	0	0	+++	+	+++	16
10^{-4} 200 organisms	1	0	0	0	0	+++	+	0	+++	++++	++++	16
	2	0	0	0	+++	+++	0	0	0	+++	++++	16
	3	0	0	+	0	0	+++	0	+++	++++	+	16
10^{-4} 2,000 organisms	1	0	+++	+	+++	++++	++++	++++	+++	++++	++++	16
	2	0	+++	+++	+++	+++	++++	+++	+++	+++	+++	29
	3	0	0	+++	+++	++++	++++	++++	+++	+++	++++	16
10^{-5} 20,000 organisms	1	+++	+++	++++	+++	+++	+++	++++	++	++++	++++	12
	2	++++	+++	+++	+++	++++	++	+++	++++	+++	++++	7
	3	+++	+++	++++	+++	+++	+++	+++	+++	++++	++++	12

Swiss mice used. Each mouse given 2 cc. intraperitoneally of a 17 hour heart's blood culture of a mouse dead with pneumococcus Type III diluted as indicated. Blood cultures taken at the intervals shown and grown in pneumococcus broth.

of a few organisms through the mucosa into the tissues and are of little, if any, import in the ultimate prognosis. On the other hand, the later cultures represent a septicemia originating in an established focus which continues to pour organisms into the blood, and, with pneumococci in mice at least, foretells a fatal outcome. The early bacteremia may not occur with strains of pneumococci that are not invasive or with organisms such as the meningococcus which does not produce disease or death when dropped into the nose. This

biphasic type of curve in the occurrence of positive blood cultures is brought out very clearly when the pneumococci are injected into the peritoneum. The protocols of such an experiment show in the higher dilutions smooth curves of a definite biphasic type with an early bacteremia, a period of freedom, and finally, a septicemia from an established focus (Table VII). It seems probable that such curves could be obtained in intranasal pneumococcus infections if the technical difficulties associated with small numbers of organisms could be overcome.

The second point is the relative lack of correlation between the degree of early bacteremia and the relative susceptibility of the breed of mice as shown by the percentage mortality and the close correlation between the mortality rate and the late septicemia, suggesting a difference in susceptibility in these mice in the tissues of the internal organs or the body fluids rather than at the body surface.

It had been thought during the early stages of this work that, since the evidence in the literature pointed against the possibility of producing pneumonia by a blood stream infection, the invasion through the upper respiratory tract must be of little importance in the pathogenesis of the pneumonia. Our own later experiments, however, have demonstrated that, provided the strain of pneumococci and the breed of mice are chosen with the same care as is used in the intranasal experiments, lesions can be produced by intravenous inoculation which resemble those produced by intranasal instillation.

SUMMARY

Unprepared mice given intranasal inoculations of certain strains of pneumococci develop pneumonia. The proportion of inoculated mice which will show the pneumonia at autopsy is dependent upon the strain and type of organism and the breed of mice used. It has been shown that, with the technique employed, the pneumococci reach the lower respiratory tract and alveoli almost immediately; moreover, that an invasion of the blood stream occurs very rapidly and can be demonstrated in a third of mice during the first 10 minutes. There is some evidence that invasion of the tissues and the blood stream may occur both through the upper respiratory tract, probably the nasal mucosa, and through the alveolar walls.

It is uncertain which route of invasion, if either, is of the most importance. It has been possible to produce pneumonia by direct intravenous inoculation of pneumococci. It may be that the pneumonia is favored by a reaction at the point of invasion through the alveolar walls in the intranasally inoculated mice, but the results of the intravenous inoculation make it clear that such a local lesion is unnecessary for the production of pneumonia in mice.

It should be stated that neither the hematogenous pneumonia nor that produced by intranasal instillation (2) has any relation to the subacute and chronic lesions of the lung found in some untreated batches of mice as a spontaneous infection. The author is thoroughly familiar with this "spontaneous pneumonia" and has studied it. Not only are the lesions described above and elsewhere (2) entirely different from those occurring in this spontaneous infection but this latter either does not occur in the inbred strains of mice used for the above studies, or occurs so rarely that it has not been seen in over 200 mice examined. Strains of mice in which the lesions occur have not been used for these pathological studies.

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EXPLANATION OF PLATES

PLATE 15

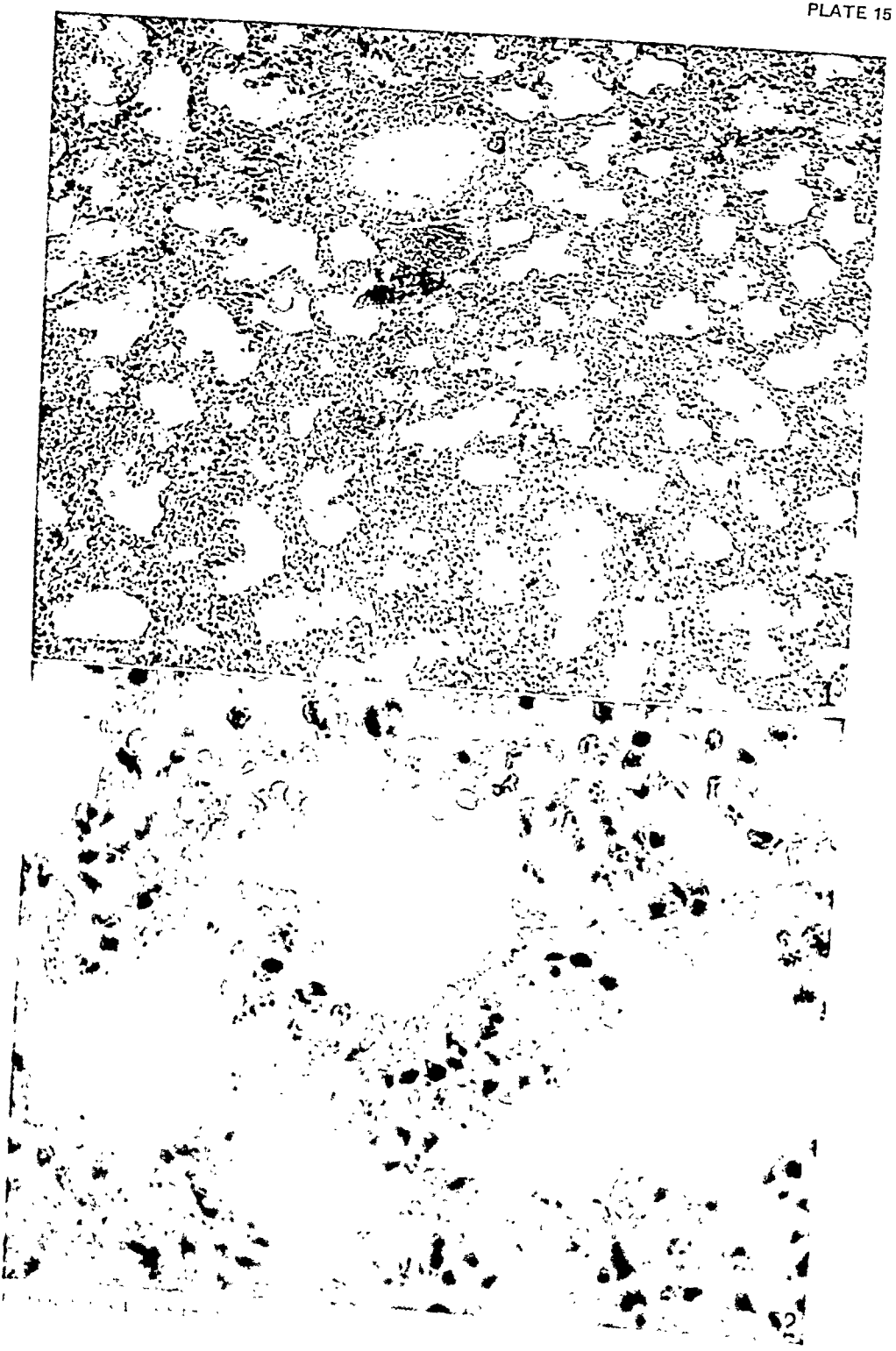
FIG. 1. Hematogenous pneumonia. Great thickening of alveolar walls wi accumulation of leucocytes and fluid outside the alveolar capillaries. Eosi methylene blue. × 100.

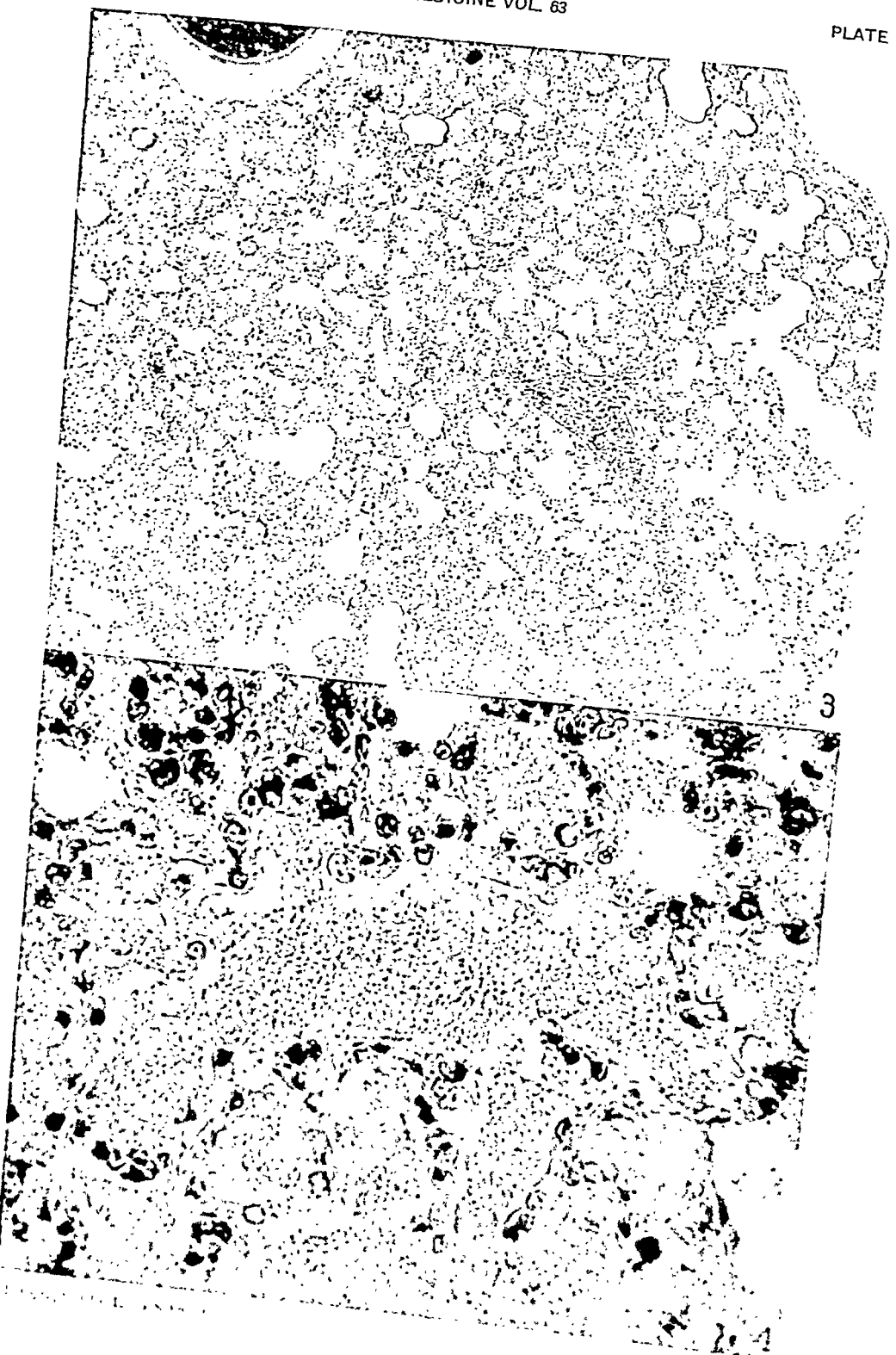
FIG. 2. Hematogenous pneumonia. Higher magnification showing infiltrati of the alveolar walls. Eosin-methylene blue. × 650.

PLATE 16

FIG. 3. Hematogenous pneumonia. The majority of alveoli in the field a filled with albuminous fluid. The exudate of cells is commencing. Eosi methylene blue. × 100.

FIG. 4. Hematogenous pneumonia. Higher magnification showing the cha acter of the exudate within the alveoli. Eosin-methylene blue. × 650.

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RESPIRATORY VERSUS GASTRO-INTESTINAL INFECTION IN POLIOMYELITIS

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The belief is general among investigators, although not unanimous, that poliomyelitis is a particular kind of upper respiratory virus infection, in which the atrium of penetration of the virus into the nervous system is the olfactory area of the nasal mucous membrane. Indeed, evidence exists indicating strongly that the virus passes both to and from the nervous organs along this path, using the axons as lines of communication in both directions.

Wickman (1), in his now classical monograph, regarded the probable portals of entry of the then hypothetical infective agent of the disease to be both the respiratory and the digestive tracts. Pathological conditions of the former structures were slight and inconstant, while the small intestine, of children especially and the lower ileum in particular, frequently showed swelling of the lymphoid apparatus, with which was associated swelling of the mesenteric nodes.

Today we are better equipped to deal conclusively with the important problem of the portal of entry of the infecting agent, because we know it to be a virus and that *Macacus* monkeys are subject to inoculation with it. We possess, therefore, an effective method of investigating the efficiency of the two channels of infection, and coincidentally, we have means of determining the presence of virus in human cases in one or the other location, as well as the capacity of the virus to survive when experimentally introduced into the monkey.

While the respiratory source of infection is generally accepted, the gastro-intestinal source is much disputed. There is, indeed, no difference of opinion on the greater ease and frequency with which infection can be experimentally induced *via* the nasal passages, or virus detected in secretions from those passages. The real question

at issue is, whether under rigid experimental conditions, infection is ever secured by way of the stomach and intestines, and whether virus occurs in these viscera at all except by accident, and is capable of surviving in them beyond the time needed for its mechanical passage through these viscera.

We may largely disregard the early experimental tests of the adequacy of the gastro-intestinal route of infection, on the grounds that they were carried out at a time when understanding of the pathogenesis of poliomyelitis was still in a rudimentary state. The case for this mode of infection has been made anew, and strongly, by Levaditi, Kling, and their coworkers (2), in a series of papers extending from 1929 to 1933; and during the past two years in a series of reports by Toomey in this country. There is no real correspondence between the experiments and views of Kling and Levaditi and those of Toomey; but they agree in the essential point of their contention that the virus does penetrate to the central nervous system by way of the digestive tract.

The experimental work on which their deductions are founded is simple in the case of Levaditi and his associates. They believe that they have discovered in *Macacus cynomolgus* a species more subject to poliomyelitis than its close relative, *Macacus rhesus*, which hitherto has been chiefly employed for experimental inoculation. While *Macacus rhesus* has not served them for feeding tests with virus, *Macacus cynomolgus* has been held to do so. The experiments themselves are performed very simply. Virus is suspended in milk or other diluent, and introduced by tube into the stomach; or it is mixed with banana and fed in the ordinary way. And in order to circumvent the stomach and the buccal and nasal cavities, it is injected directly into the intestine of laparotomized animals.

The actual number of experiments carried out on *Macacus cynomolgus* was not large, but the high proportion of successful tests makes up for the limited number of feedings. Of seven *cynomolgi* fed by tube or otherwise, six developed paralytic poliomyelitis. Two of this species received direct, syringe injections into the small intestine, from which one did and the other did not become paralyzed. And, finally, of three *Macacus rhesus* inoculated intracerebrally with mesenteric nodes removed from three of the fed paralyzed monkeys, two responded with typical poliomyelitis.

The conclusions drawn by them from these tests are sufficiently obvious: *Macacus cynomolgus* is highly susceptible to fed virus and also to virus injected directly into the intestine; from the intestine the virus penetrates to the central nervous system along two channels—nerves and lymph (ultimately to the blood). No explanation is offered as to the way the virus reaches nerve channels through the intact mucous membrane. A single experiment made many years ago by Landsteiner and Levaditi, in which virus was injected directly into the mesenteric vein, is cited to show that it is possible for lymph or blood to carry virus to the central nervous organs under circumstances leading to the paralytic disease.

The theoretical and practical significance of these experiments is so large that they were promptly repeated by Clark and his associates (3), who failed to confirm them, and by Dr. C. P. Rhoads, whose results also were negative. Rhoads went a step further and subjected the previously fed monkeys to nasal instillations of virus; the animals responded with typical symptoms of paralytic poliomyelitis.[1] This test did away with the argument of individual lack of susceptibility advanced by Levaditi to explain the failure of certain feeding tests.

Recently Toomey (4) has attacked anew the question of the gastro-intestinal portal of entry of the virus, and has devised experiments which give characteristic positive and negative infection results. He has, however, departed widely from the methods previously employed which sought to do the minimum degree of damage to the intestinal structures and to maintain them, at the time of inoculation, in a state of physiological function. Toomey's manner of approach may, therefore, be called drastic. He clamped off sections of the small intestine and filled them to ballooning with suspensions of virus; infection sometimes followed. Or he injected virus into the subserosa; here, again, paralysis sometimes occurred. And, finally, he combined virus with a toxic filtrate of colon and other intestinal bacteria, and found such mixtures even more effective in producing paralysis in the overdistended, constricted gut or the inoculated serosa, than virus alone.

Toomey's experiments are founded on the long known fact that whenever virus comes into close relationship with nerve fibrils, infection may occur. Now, unmyelinated fibers, the most readily entered by the virus, are embedded in the intestinal wall. It is apparent that the particular techniques Toomey employed favor the making of the necessary contact. It matters little whether the path to the nerve fibers is supplied by mechanical means or by the use of a chemical agent, such as the "enteric toxin," which is not an indifferent substance, as shown by the "diarrhea and anorexia" it provokes. Such bacterial extracts have long been known to produce focal necrosis and hemorrhage in the intestine, and they in turn provide the breaks in continuity favoring the passage of virus into the submucosa, where unmyelinated fibers abound.

It may, therefore, be questioned whether Toomey's experiments do more than bring new evidence to support the impenetrability of the intact mucosa of the intestine to the virus. It is probably true, as he holds, that virus injected directly into the gut, after laparotomy, is swept onward; and yet, success has not been wanting to this class of experiments, as shown by the early ones of Leiner and von Wiesner, and the recent experiments of Kling and Levaditi. The explanation of the success is to be sought probably in the contamination, in the course of the needle puncture, of the wall of the intestine containing nerve fibers. That fed virus is not swept rapidly through the gut is revealed by extraction tests made with the feces; adequate time is afforded for the contact of vast amounts of virus with the mucous membrane, and yet when precaution succeeds in keeping virus away from the buccal and nasal cavities, symptoms of infection fail to arise.

[1] These experiments, although carried out in 1929, have not been previously reported.

EXPERIMENTAL

Feeding Tests on Cynomolgi.—Rhoads' feeding experiments departed in detail from those of Kling and Levaditi, and wholly in that he later nasalized with the same strain of virus the monkeys which had failed to react to the fed material. The table which follows summarizes the tests.

TABLE I

Rhoads' Feeding Tests in Macacus cynomolgus, 1929

Monkey	No. and date of feedings	Amount of feeding	Mixed Virus suspensions	Interval	Preliminary treatment	Result
	1929	*cc.*	*per cent*	*wks.*		
Cynomolgus						
1	9, Nov. 1	25	20	1	$NaHCO_3$ + opium	Died of intercurrent disease
2	9, " 1	25	20	1	" " "	No symptoms
3	2, Dec. 12	12	20	2	" " "	" "
4	2, " 12	12	20	2	" " "	" "

Of these four monkeys, one died of intercurrent disease; the others remained free from all symptoms for an indefinite period of time. After a rest period of 4 or more weeks, the survivors were given nasal instillations of 10 per cent glycerolated virus; they all showed symptoms on the 8th to the 12th day after the first instillation. The symptoms were abortive in one monkey, and they progressed to paralysis and death in the other two animals. Leg paralysis first manifested itself in one, and face and eyelid paralysis (double ptosis) in the other. These results speak for themselves.

The severe epidemic of poliomyelitis of 1931 led us to reexamine the question of the gastro-intestinal portal of entry of the virus. We proceeded in a somewhat more comprehensive manner, studying the effects of tube feedings in ways simulating those employed by Kling and Levaditi, and searching anew for the virus in the intestine and the mesenteric nodes of human victims succumbing to the acute disease. As will be observed in Table II, three *cynomolgi* were tube fed, and three dropper fed. The latter animals were held in a semirecumbent position, and the suspension was dropped slowly into the mouth, time being allowed for swallowing. The three abundantly tube fed

monkeys all remained well. It is noteworthy that the only animal developing symptoms was dropper fed. The chances of buccal and nasal contamination are obviously greater in dropper than in tube feedings.

TABLE II

Feeding Tests in Macacus cynomolgus, 1931

Monkey	No., date, and manner of feedings	Amount of feeding	Mixed Virus suspensions	Interval	Results
	1931	*cc.*	*per cent*		
Cynomolgus					
1	7, Sept. 28, stomach tube	180	10	Daily	No symptoms
2	7, " 28, " "	180	10	"	" "
3	7, " 28, " "	410	10	"	" "
4	7, " 28, dropper	180	10	"	" "
5	7, " 28, "	210	10	"	" "
6	7, " 28, "	180	10	"	*Left leg flaccid; right leg partially paralyzed. Sacrificed for histology

* The lesions, typical of poliomyelitis, were widespread (5).

The surviving, symptomless *cynomolgi*, 1 to 3 months after the feedings, were instilled on 6 successive days with suspensions of the same strain of virus used in the feedings. 4 to 7 days after the last instillations, four animals manifested symptoms; three developed widespread paralysis; the fourth passed through an abortive attack of poliomyelitis. It succumbed to a second course of instillations in a typical manner. One monkey escaped infection.

Four control monkeys were used in the nasal test—two *cynomolgi* and two *rhesi*. One of each pair became paralyzed. The previously fed *cynomolgi* proved, therefore, at least as subject to infection by the nasal route as the control animals.

To these two sets of essentially negative feeding tests made with *Macacus cynomolgus* should be added a third carried out at a later date. This experiment was made in order to examine the mesenteric nodes for virus, since Kling and Levaditi had been successful in producing paralytic infection with nodes taken from fed animals. The mesenteric nodes in *Cynomolgus* A and B were removed surgically. After recovery and a proper interval, the nasal instillations were given.

TABLE III

Feeding Tests in Macacus cynomolgus, 1934–1935

Monkey	Dates of tube feedings	Amount of feeding	Virus suspensions	Results
		cc.		
Cynomolgus A	Oct. 15, 1934	5	25 per cent Mixed Virus	No symptoms
	" 20, 1934	5	25 " " " "	" "
	" 25, 1934	10	25 " " " "	" "
	Feb. 16, 1935	25	25 " " Philadelphia, 1932, virus	" "
Cynomolgus B	Oct. 15, 1934	10	25 per cent Mixed Virus	" "
	Feb. 16, 1935	25	25 " " Philadelphia, 1932, virus	" "

TABLE IV

Nasal Tests in Macacus cynomolgus, 1935

Three instillations of Philadelphia, 1932, virus given on Mar. 19, 21, 25, 1935

Monkey	Cells in cerebrospinal fluid		Results
	Dates	Cells	
	1935		
Cynomolgus A	Mar. 19	16	Temp. 103–103.4°F.
	" 21	24	
	" 25	27	
	" 27	40	
	" 29	420	Excited; globulin ++; Temp. 104.8°
	" 30	—	Paralysis; Temp. 105.8°
	Apr. 1	—	Prostrate; " 103.4°
Cynomolgus B	Mar. 19	21	Temp. 102.6–103°F.
	" 21	20	
	" 25	29	
	" 27	185	Temp. 104°
	" 29	460	" 107°. Tremor; ataxia
	" 30	—	Paralysis
	Apr. 2	—	Prostrate

Comments.—The feeding tests carried out by Rhoads and ourselves from 1929 onwards confirm those of Clark and his associates; and they stand in sharp contrast to those of Kling, Levaditi, and their co-workers. They are perhaps more convincing because they are more complete than any similar tests previously made, by reason of the comparisons made on the fed animals with respect to nasal susceptibility to the same strain of virus. The contrast is very impressive and brings into sharp relief the fact of the impenetrability of the normal intestinal mucous membrane to virus present in enormous amounts, at the same time that the fed monkeys are capable of taking up readily, by way of the olfactory area of the nasal mucous membrane, far smaller quantities of virus, which induce characteristic experimental poliomyelitis.

We may now summarize the results as given by Kling and Levaditi and obtained by ourselves. The former fed seven *cynomolgi*, of which six became paralyzed; we fed eleven, of which ten remained symptomless. Of ten of the monkeys of the latter series, subjected later to nasal instillation, nine responded with symptoms (and lesions) of the disease. We believe, therefore, that when paralysis arises in the course of the feeding experiments, contamination of the buccal and nasal membranes with virus may be considered to have taken place.

Direct Intestinal Injection of Virus

Leiner and von Wiesner (6) found in 1910 that laparotomized *Macacus rhesus*, although resisting direct feedings, would respond with paralysis to virus directly (needle) injected into the small intestine. This particular experiment has been repeated from time to time with contradictory results. Kling and Levaditi performed it on *cynomolgi*, in keeping with and in support of their belief that this species of *Macacus* is more subject to infection by the intestinal route. Of two *cynomolgi* injected by them (on both the needle puncture was seared with cautery), one remained well and one became paralyzed. Clark and his associates injected four laparotomized *cynomolgi*: two died of intercurrent disease; the other two remained well. The results obtained by us in seven tests, in which direct intestinal injection of virus was made, are summarized in Table V.

Comments.– In the series of experiments summarized in Table V

TABLE V

Direct Intestinal Injection of Virus, 1934–1935

Monkey	Date of inoculation	Weight	Virus suspensions	Results
	1934	*gm.*		
Cynomolgus				
1	Nov. 19	1600	1 cc. 25 per cent Mixed Virus	No symptoms
	1935			
2	June 27	1675	25 " 25 " " Philadelphia	" "
3	" 27	1360	5 " 25 " " "	" "
4	Mar. 21	1885	25 " 25 " " "	Mar. 28, Temp. 102.4°F.
				Mar. 29, 10 a.m., Temp. 104.6°. 5:30 p.m., 105.4°, ptosis, tremor
				Mar. 30, dead. Typical lesions; puncture wound closed; hemorrhage into intestinal wall
5	" 21	1700	25 " 25 " " "	Apr. 8, Temp. 105.4°, 524 cells in cerebrospinal fluid
				Apr. 9, ptosis; tremor
				Apr. 10, death, respiratory failure. Typical lesions
Rhesus				
6	May 3	1650	25 " 25 " " "	No symptoms
7	" 3	1580	5 " 25 " " "	" "

attention is directed to Monkeys 3 and 7, which were chosen because they were smaller and younger than those usually employed for experiment. Of the two *cynomolgi* becoming paralyzed, Monkey 4 proved informing. Microscopical examination of the injection site, which to gross inspection was healed, showed hemorrhage into the intestinal wall, a finding which supported our belief that in the making of such injections of virus, the latter may readily be introduced into the tissues, as well as into the intestinal lumen. It may be regarded

as the more remarkable that these drastic inoculations do not lead more often to symptomatic effects. An illustration of the capriciousness of this general class of experiments is to be found in Monkey 1. On February 16, 1935, this *cynomolgus* was given by tube 25 cc. of a 25 per cent salt solution suspension of Philadelphia virus. On March 7 (19 days after the feeding) the animal was tremulous and ataxic, and showed deltoid paralysis. The cerebrospinal fluid contained 434 cells and the globulin was +. Eventual recovery with residual arm paralysis ensued.

Mesenteric Nodes

Kling and Levaditi, in their support of the invasion of the virus from the digestive tract, revived the question of the infectivity of mesenteric nodes. In two instances—one after feeding, the other after injection of virus into the intestinal lumen in *cynomolgi*—they induced paralysis by inoculating the nodes cerebrally into *Macacus rhesus* monkeys. This result led them to postulate a lymphatic and eventual blood carriage of virus to the central nervous system, in addition to the recognized, more direct, neural route. They offer two experiences in support of this point of view, one being the successful inoculation by Flexner and Lewis (7) of mesenteric nodes removed at human autopsy, and the other the injection of virus by Landsteiner and Levaditi (8) into the mesenteric vein of *Macacus* monkeys.

These early tests scarcely suffice today, in view of the greater stringency of the experimental technique. The node removed at autopsy in 1910 was taken in the course of the ordinary procedure, without the use of sterile instruments; in making the intravenous injection, the danger of perivascular nerve contamination was not regarded.

We have, therefore, restudied this subject, employing for inoculation mesenteric nodes removed aseptically at autopsies performed on acutely fatal human cases, before the brain and spinal cord were exposed, and from fed *Macacus rhesus* and *cynomolgus* monkeys. We have gone beyond the mere testing of the nodes and have tested also the intestinal mucosa of the lower ileum, in which the lymph nodes are hypertrophied. The 1931 epidemic of poliomyelitis provided us with nodes from three autopsies; the inoculations were made with the fresh nodes and with glycerolated specimens for acceleration. Eight *rhesi* were injected cerebrally with this material; symptoms did not arise in any instance. In three of the eight animals, nodes from single human cases, and in five the pooled nodes from all three, were employed as inocula. During the recurring outbreaks of poliomyelitis

in New York from 1911 to 1913, nodes from five acutely fatal cases were injected cerebrally, peritoneally, and into the sciatic nerve; in no instance was disease produced.

The mucosa of the ileum containing the enlarged nodules was scraped off, suspended in salt solution, after being ground with sand, shaken, centrifuged, and filtered through Berkefeld candles, the filtrate being concentrated *in vacuo* by Clark's method and injected cerebrally and peritoneally into three *rhesi*. No symptoms arose. These negative tests confirm a larger series made in 1911 to 1913 with intestinal mucosae rendered bacteria-free with 0.5 per cent phenol.

Two recent sets of experiments designed to show whether virus introduced into the stomach by tube, or into the intestine by needle injection, passes in detectable quantities into the mesenteric nodes, are summarized in the following protocols.[2]

Protocol I. Macacus rhesus.—(1) 50 cc. of a 20 per cent suspension of virus tube fed; after 3 hours, mesenteric nodes removed surgically;[3] 5 days later additional nodes excised. Suspensions of each set of nodes were injected cerebrally and peritoneally into a *Macacus rhesus*. No symptoms developed.

(2) 100 cc. of a 20 per cent suspension of virus tube fed; second similar feeding 4 hours later. 7 hours after first feeding, nodes removed surgically and injected cerebrally and peritoneally into a *Macacus rhesus*. No symptoms followed. At the time of the second removal of nodes, rectal washings made and the fluid passed through a Berkefeld candle; filtrate injected cerebrally and peritoneally into two *rhesi*. Both became paralyzed; spinal cord lesions typical.

(3) Two *rhesi* were fasted, laparotomized, and each given 80 cc. of a 20 per cent suspension of virus into the ileum. 6 hours after operation, nodes were excised surgically and injected cerebrally and peritoneally into two healthy *rhesi*. No symptoms arose either in the monkeys given mesenteric node injections or the injections into the gut.

Protocol II. Macacus cynomolgus.—(1) Three tube feedings of 5, 5, and 10 cc. of a 25 per cent suspension of virus given a *cynomolgus*. 11 days after the first feeding no symptoms had arisen. Nodes were removed and injected cerebrally; 7 days later an accelerating injection given. No symptoms appeared in fed or injected animals.

(2) *Cynomolgus* laparotomized and given heavy suspension of virus by direct, needle, injection into ileum. 11 days later nodes were excised and injected cerebrally into a *rhesus*; accelerating injection given 7 days later. No symptoms arose in either animal.

(3) 10 cc. of heavy suspension of virus tube fed. After 11 days, removed

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[2] Ether anesthesia was employed in all operative procedures.

[3] I wish to thank Dr. Harold L. Amoss for carrying out the operative procedures.

nodes injected cerebrally into a *rhesus*; accelerating dose 7 days later. No symptoms appeared in either animal.

(4) 25 cc. of a 25 per cent suspension of virus injected into ileum of laparotomized *cynomolgus*. Symptoms of poliomyelitis appeared on the 19th day, death ensued on 21st day. The excised nodes were injected twice: immediately at the time of autopsy, and 7 days later. No symptoms arose.

Comments.—The larger series of negative tests given with mesenteric nodes and intestinal mucosa from acutely fatal human cases, and from the tube fed and needle injected monkeys—both *Macacus rhesus* and *cynomolgus*—carry their own interpretation. They stand in sharp contrast to the early, successful inoculation of human mesenteric nodes reported by Flexner and Lewis, and the recent ones of Kling and Levaditi. We believe that the original experiment of Flexner and Lewis was faulty in technique; we have no explanation to offer for the positive inoculations reported by Kling and Levaditi. There is one unequivocal report among a number of dubious ones, of the finding of the virus in the intestinal washings of a fatal human case. This case, reported by Kling, Pettersson, and Wernstedt (9), takes on additional interest because the tracheal washings were also infectious. It is open to conjecture whether in this exceptional instance, virus from the nasopharynx was not both swallowed and aspirated in considerable amounts during the last hours of life.

Intravenous Inoculations

Kling and Levaditi cited an earlier successful injection of virus into a mesenteric vein as indicating the occurrence of a lymph-blood conveyance of the infectious agent to the central nervous system in the monkey. In this way the significance of the demonstration of virus in the mesenteric nodes of fed monkeys was emphasized. The comprehensive series of tests made by Flexner and Amoss (10) would seem to negative this point of view; and the failure by us to induce poliomyelitis by the injection of mesenteric nodes of man and the monkey, and of the mucosa of the ileum of man, contrasts with the two successful experiments of Kling and Levaditi.

In referring to the instance in which Landsteiner and Levaditi induced paralysis by virus injected into the mesenteric vein, we stated that no account had been taken of the perivascular nerves which, if contaminated, would convey the virus to the spinal cord. We have

tested this point in two ways, of which one incidentally again brin the vascular route under examination. In one experiment fo *Macacus rhesus* monkeys were injected intravenously on 7 successiv days with 5 cc. of a 5 per cent suspension of virus, by way of the saph nous vein. Three control *rhesi* were given nasal instillations an equ number of times. The controls became paralyzed on the 10th, 11t and 17th day respectively. Three of the four intravenously inocu lated monkeys remained well, the fourth becoming partially paralyze as described in the following protocol. In the other experiment, viru was injected directly into the vascular wall.

Protocol. Macacus rhesus.—June 10 to 16, 1931, seven daily intravenous inje tions of 5 cc. of 5 per cent glycerolated suspension of Mixed Virus. June 2 tremor; ataxia; right deltoid paralyzed; legs very weak. June 26, right ar paralyzed; legs almost paralyzed; sits up. July 1, improved. Oct. 2, complet recovery.

This experiment is virtually a confirmation of the earlier result obtained by Flexner and Amoss. It is, however, desirable to accoun for the occurrence of paralysis in the one monkey of the four. Th injection by syringe of the virus into the lumen of the vein cannot b carried through in all instances without some degree of contaminatio of the vascular wall and contained nerve fibers. Hence two monkey received injections of virus directly into the coats of the expose saphenous vein, where it would come into close relation with nerv fibrils.

There is still another point to be considered. Toomey (11) ha emphasized the unmyelinated nerve fibrils in the skin in order to ac- count for the occasional paralysis arising during active immunizatio of virus intradermally. The like behavoir of the unmyelinated fiber in the intestinal wall and the skin is brought together by him in this way.

Since it is not feasible to make successive inoculations into the sub- mucosa, it was decided to study also the cerebrospinal fluid in monkeys receiving multiple intradermal injections of virus. A deduction could be made of the ease with which the nerve fibrils in these two locations carry virus to the center as compared with the olfactory areas in the nasal membrane.

Protocols. Saphenous Vein Experiment.—Two *Macacus rhesus* monkeys were operated under ether anesthesia and aseptic technique. The long saphenous vein

in the right side was exposed for 3 or 4 inches. 5 per cent suspension of glycerolated Mixed Virus was injected at several points into the perivascular tissues and muscularis; blebs were raised in the latter. The cells in the cerebrospinal fluid were counted and temperatures taken.

Monkey A			Monkey B		
Dates	Temperatures	Cells	Dates	Temperatures	Cells
1935	°F.		*1935*	°F.	
Oct. 11		19 (normal)	Oct. 11		9 (normal)
" 12	103.4	25	" 12	102.8	14
" 14	103	57	" 14	103.2	18
" 16	104.2	25	" 16	103.8	45
" 18	104	12	" 18	104.2	115
				Monkey excited	
" 21	102.8	14	" 21	103	52
				Less excited	

Comment.—Both Monkeys A and B responded to the virus injected into the vascular coats. The effect on the cerebrospinal fluid of Monkey A was rapid, but transitory, and no clinical symptoms appeared; while the effect on the cerebrospinal fluid of Monkey B was more slowly developed, but more pronounced. This animal had what was probably a mild abortive infection. Hence the virus actually passed from the coats of the vessels along nerve fibers to the center, as had been surmised in the monkey (page 220) responding to the repeated intravenous inoculations.

Skin Experiment.—Two *rhesus* monkeys were given ten intradermal injections of 2 cc. each of 5 per cent Mixed Virus at 3 day intervals. Temperature readings and cell counts in the cerebrospinal fluid were made.

Monkey C			Monkey D		
Dates	Temperatures	Cells	Dates	Temperatures	Cells
1935	°F.		*1935*	°F.	
Sept. 14		2? (normal)	Sept. 14		24 (normal)
" 16	102.8	17	" 16	102.4	25
" 19	102.4	22	" 19	102	29
" 21	103.2	27	" 21	103.8	23
" 26	104	24	" 27	103	19
" 30	103.2	45	" 30	104	70
Oct. 3	103	37	Oct. 3	103	15
" 10	102.8	31	" 10	103	22

Comment.—The reactions in the cerebrospinal fluid from successive intradermal injections of virus were negligible. A transient increase in the cell count occurred in Monkey D; it was unattended by clinical symptoms. The nerve fibrils in the skin are obviously much poorer conductors of virus to the center than are those of the olfactory areas in the nasal membranes. This fact could be inferred from the great differences in clinical symptomatic response from the two kinds of inoculations. Not improbably the unmyelinated fibers in the wall of the intestine are also relatively poor conductors, as compared with the olfactory nerves.

Nerve Conduction

That the virus of poliomyelitis has an especial affinity for nerve structures and tends to travel along the axons is generally believed. No other virus is known to be so strictly neurophilic. Although a more sensitive indicator than the monkey might reveal it in the general tissues and humors of the body, at present we are ignorant of its affinity for these tissues or its capacity to survive in blood or lymph. Its absence from the cerebrospinal fluid under circumstances in which it is abundantly present in the adjacent, damaged cells of the spinal cord and brain, is noteworthy (12). One set of conditions is known to exist in which at least survival occurs in organs remote from the central nervous system. Flexner and Amoss (13) injected large amounts of virus intravenously into a *rhesus* monkey; no symptoms followed, and when the animal was etherized 17 days later, a suspension of the spleen proved infective when inoculated cerebrally into another monkey. At the same time the spinal cord and medulla were without effect.

On the other hand, virus introduced into and sealed in the brain passes into the nasal mucous membrane and its secretions, as far as known, only along the olfactory nerve filaments; it also passes, as Flexner, Clark, and Amoss (14) showed, along nerve channels to the abdominal sympathetic ganglia. The virus of poliomyelitis, therefore, shares with the viruses of rabies and Borna disease, the property of wandering long distances along nerve fibers. Tests for virus in the abdominal sympathetic ganglia were carried out again in 1933. As the protocols which follow show, the presence there was again demonstrated.

Protocol. Experiment A.—Dec. 8, 1933, *Macacus rhesus* given double (intracerebral and intraperitoneal) injections of suspension of abdominal sympathetic ganglia taken from two *rhesi* acutely paralyzed after cerebral inoculation of virus. Dec. 18, tremor, left facial and leg paralysis. Dec. 20, dead.

Experiment B.—Dec. 20, 1933, sympathetic ganglia from Monkey A doubly injected into a *Macacus rhesus*. On the 8th day a second (acceleration) inoculation of the ganglia from an acutely paralyzed monkey. Slight, evanescent symptoms lasting 2 days followed the accelerating inoculation. Result doubtful.

Elimination of Virus

Intestinal.—The manner of elimination of the virus from the digestive tract has a bearing on the disputed question of the gastro-intestinal portal of infection. Levaditi, Kling, and Lépine investigated this subject, with results so irregular that interpretation is made difficult or impossible. Their methods of procedure were not such as to make success probable, as they employed for inoculation unconcentrated filtered extracts of the fecal discharges of fed monkeys or of artificial mixtures of feces and virus. Clark and his associates applied the effective concentration and dialysis method they devised to a search for virus in the dejecta from the intestine. Their positive results may be regarded as conclusive, and the slight irregularity in them such as would be expected to arise from the complex materials subjected to extraction and the small number of monkeys inoculated with the concentrates. In brief, their findings show that after direct intestinal injection of virus, infective doses may be recovered from the feces; that, similarly, the feeding of virus suspensions or filtrates for several days, is followed by the elimination of virus in a viable and infective state; and also that the mere feeding of large quantities of filtrate for one day, suffices to give to the dejecta effective properties.

Dr. Henry W. Scherp has investigated this subject, in a somewhat more extensive manner, using the Clark concentration method of preparing the extracts. The results obtained are given in the protocols that follow.

Protocol 1. Macacus rhesus.—Two monkeys were fed by stomach tube 30 cc. of a 10 per cent milk suspension of Mixed Virus on 2 successive days; feces collected from 6th to the 30th hour after second feeding. The extracted filtrates, concentrated and dialyzed, were inoculated into two *Macacus rhesus* by intracerebral and intraperitoneal injections. Both inoculated monkeys became prostrate on the 7th day. The fed animals remained well.

Protocol II. Macacus rhesus.—Two monkeys received 30 cc. of a 10 per cent milk suspension of Mixed Virus by stomach tube on 2 successive days. Feces were collected from each at 24 and 96 hour intervals and 5 days after second feeding. The concentrate from each was injected intracerebrally and intraperitoneally into *Macacus rhesus*. The 24 hour fecal specimens only yielded infective filtrates; the animals receiving the concentrates prepared with the 96 and 120 hour specimens remained well. The fed animals showed no symptoms.

Protocol III. Macacus cynomolgus.—Fed by stomach tube on 4 successive days with 30 cc. of 10 per cent suspension of virus in milk. Feces collected 24 hours and 5 days after last feeding. Concentrates prepared from each specimen, and inoculated intracerebrally and intraperitoneally into *Macacus rhesus* monkeys. The animal receiving the 24 hour specimen became paralyzed; the animal receiving the 5 day specimen remained well. The fed animal remained well.

Although the tests represented in the protocols are few in number, they are consistent in showing that when large feedings are given and the virus reaches the intestine in an active state, it fails to attach itself to the mucous membrane in a way leading to prolonged elimination or increase in amount. The gut is not a favorable locus for the virus. Under the circumstances it is not, therefore, among other things probable, as Kling and Levaditi would have it, that virus escaping from the intestine may contaminate potable water supplies, be thus widely distributed, and result in water-borne epidemics of poliomyelitis (15).

Nasal and Buccal Membranes

The presence of virus in the secretions or in the substance of the nasal and buccal mucous membranes in man and the monkey has been established beyond all doubt (16). The detection of virus in these locations is difficult, because of inadequacy of methods of securing it in sufficient concentration for favorable inoculation. Scherp tested the Clark concentration method on the excised mucous membranes of paralyzed monkeys. Three tests were made. In one the virus had been injected cerebrally; in two, nasal instillations had been given. Two of the three tests were successful, in that virus in infective quantities was recovered—one from the cerebrally inoculated, and one from nasally instilled monkeys. The extracts contained a troublesome amount of mucus which made filtration difficult. The results are given in the protocols, since they may have interest in leading to a more effective way of finding virus in the upper respiratory membranes.

Protocol I. Macacus rhesus.—Given two double (cerebral and peritoneal) injections of concentrate prepared from two *rhesi* cerebrally injected with Mixed

Virus, paralyzed on the 7th and 8th day respectively. The first inoculations were made on Dec. 24, 1931, and the second (accelerating) on Jan. 1, 1932. 7 days after the second injections there were tremor, ataxia, and right arm paralysis. 2 days later the monkey was moribund. The lesions were typical of poliomyelitis.

Protocol II. Macacus rhesus.—Doubly injected with concentrate prepared from two monkeys which became paralyzed following six daily nasal instillations of Mixed Virus, one on the 10th and the other on the 14th day after the first instillation. The first injections of concentrate were given on Dec. 15, and the second (accelerating) on Dec. 23, 1931. 7 days after the second inoculation there were present tremor and leg paralysis. The lesions in the spinal cord were characteristic.

SUMMARY AND CONCLUSION

The debated problem of gastro-intestinal *versus* respiratory mode of infection in poliomyelitis has been restudied by several investigators recently, with conflicting findings. Kling and Levaditi in Europe carried out experiments from 1929 to 1933, which led them to the conclusion that the digestive tract affords a ready entrance of the virus of the disease into the body. They believe that the substitution of *Macacus cynomolgus* for *Macacus rhesus* as the animal of choice for the tests supports this point of view. Toomey in the United States has arrived at a similar conclusion, not by employing a particular species of monkey for experiment, but by the use of drastic measures of inoculation, which insure that the virus makes contact with the unmyelinated nerve fibers embedded in the intestinal wall. Toomey's methods are so severe and artificial that his results cannot be regarded as simulating a natural mode of infection.

We have repeated the tests of Kling and Levaditi, but in a far more comprehensive manner than was followed by them, and, like Clark and his associates who early repeated them, we have failed to confirm them. Indeed, we do not find *Macacus cynomolgus* and *rhesus* to differ in any essential way in their response to the presence of the virus of poliomyelitis in the body. *Cynomolgi* do not respond to virus introduced into the stomach when contamination of the buccal and nasal cavities is avoided; they respond, as do *rhesi*, to virus directly injected into the intestine when virus passes into the intestinal wall and makes the necessary nerve fiber contact. Both *Macacus cynomolgus* and *Macacus rhesus* which have resisted feedings of virus are subject to nasal instillations of the same strains of virus and in the same degree.

On the basis of the experiments reported in this paper we can reaffirm the conclusion previously arrived at by ourselves, and confirmed

independently by investigators in Europe and America, namely that the only established portal of entry of the virus of poliomyelitis into the central nervous system of man is the nasal membrane, and especially the olfactory nervous areas in that membrane.

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A STUDY OF GENERALIZED VACCINIA IN THE CHICK EMBRYO*

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PLATES 17 TO 19

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Numerous investigators during the past thirty years have conclusively demonstrated that in cutaneous vaccinia of experimental animals lesions of the disease may be generalized throughout the organism. Animal inoculations with histological studies of the different organs from an infected animal have established that, besides the manifestation of a local cutaneous lesion, the virus is quite generally distributed during the course of the disease.

It is not intended in this report to review the extensive literature which exists on this subject. Several excellent recent reviews,—those by Sobernheim (1), Arnold (2), Gins (3) and Lillie and Armstrong (4),—can be referred to by those interested in this aspect of the subject.

We wish herein to present our observations on the behavior of vaccine virus when inoculated on the chorio-allantoic membrane of the developing chick with special reference to the distribution of the virus throughout the embryo itself. In the course of our investigations on the use of the embryonic membranes of the chick for the propagation of the virus of vaccinia (5, 6) it was frequently noted that macroscopic lesions occurred in the liver and spleen of chick embryos thus infected. It was also observed that an occasional chick which survived the infection longer than 3 or 4 days developed a generalized eruption of the skin.

This led to the investigation of the distribution of the virus throughout the organism of the embryo and to a histopathological study of

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the lesions in the different organs. Since vaccine virus can be easily propagated in pure culture in the chick embryo (5) it seemed that such a study would be of special interest.

The Distribution of Vaccine Virus in the Various Organs of the Chick Embryo

A large number, usually 24 to 30, 14 day old, chick embryos were inoculated on the chorio-allantoic membrane by the method described elsewhere (6). Small pieces of membrane previously infected with a strain of dermal vaccine which had been propagated in pure culture in the membrane through 100 generations were used as inoculum. As very few embryos survive the infection following the method of inoculation longer than 72 hours, all those which were still living at the end of this period were aseptically removed from the egg. The membranal lesion was first removed to lessen contamination of the embryo from this source. The organs from at least 10 or 12 infected embryos were used for each experiment. They were carefully removed and kept in separate sterile containers to prevent cross-contamination as much as possible. At the 72 hour period the liver and spleen of all embryos showed definite evidences of involvement by the presence of macroscopic areas of focal necrosis. The liver, spleen, kidneys, heart, brain, gastro-intestinal tract, bone marrow represented by the femurs, large areas of skin over the back and the membranal lesions were removed and the individual organs and tissues pooled in separate sterile containers. These specimens were then carefully washed in saline and ground finely in separate sterile mortars. From the ground pulp dilutions by volume of 1–100, 1–1000, 1–10,000, 1–30,000 and 1–50,000 were set up in sterile saline. From each dilution of the various organs 0.2 cc. was inoculated into an area 2.5 cm. square on the shaved and scarified rabbit skin.

The results from several of such experiments were remarkably uniform and the following tabulation represents averages thus obtained.

Organs	Dilution				
	1–100	1–1000	1–10,000	1–30,000	1–50,000
Membrane	+++++	++++	++++	+++	+
Liver	+++++	++++	++++	+++	+++
Spleen	++++	+++	+	+	−
Bone marrow	++++	++++	++++	++	+
Skin	++++	++++	+	−	−
Gastro-intestinal tract	++++	+++	+	+	−
Kidney	++++	++	+	−	−
Heart	+++	++	−	−	−
Brain	+	−	−	−	−

These results show that in the infected chick embryo the virus is distributed throughout the entire organism. It is of interest to note that the liver possesses the highest titratable infectivity. Whether there is quantitatively more virus in the liver than in the membranal lesion is difficult to determine because the liver tissue is so much more readily ground up finely than the membrane. A better dispersion of the virus in the higher dilutions of the parenchymatous organs may be expected. The bone marrow also shows itself to be rich in virus. There is no definite evidence for a predilection of the virus for the skin. It is also quite noteworthy that very little if any virus was found in the brain tissues. Such virus as was present could be accounted for as being contained in the blood in that organ. The titration of the heart tissue reveals a fair amount of virus present in this organ, and as will be seen from the histological study this represents an actual infection and is not to be accounted for altogether by the presence of the virus in the blood stream.

The quantitative relationship of the dispersion of the virus throughout the various organs as is roughly estimated by these experiments seems to indicate that mechanical or physical factors mainly determine the amount of virus distributed to the various organs. The severity of the resulting infection is evidently further determined by direct access to and the ability of the virus to multiply in the cells of the various organs.

The Pathology of Generalized Vaccinia in the Chick Embryo

Methods

Sixty 12 day old chick embryos were inoculated on the chorio-allantoic membrane with a dermal strain of virus which had been passed in pure culture in the membrane through 100 generations. At 24 hour intervals 2 or more embryos were removed from the eggs. The younger embryos up to 15 days of age were fixed *in toto*. The organs from embryos older than this were removed separately and fixed in Zenker's solution (10 per cent acetic acid) and in corrosive sublimate alcohol mixture (Gins (7)). Gross pathological descriptions of all the organs were carefully recorded. The tissues were embedded in paraffin, stained with hematoxylin and eosin, cleared in xylol and mounted in balsam. It was found that the tissues fixed in corrosive sublimate and alcohol gave the most satisfactory results. The cellular and intracellular structure of the tissues fixed in this manner could be excellently demonstrated when stained with hematoxylin and eosin. Normal chick embryos of corresponding ages were treated in the same manner.

In this way a series of infected embryos was collected and sectioned, representing daily stages in the disease from the 1st through the 7th day after inoculation.

Smears were also made from the lesions in the various organs and stained by the Morosow technique (8) in an effort to demonstrate the presence of Paschen corpuscles.

Gross and Microscopic Pathology of the Lesions in the Various Organs of the Chick Embryo

Liver.—At 24 hours the liver shows very little change except a slight swelling and a deeper red color than normal. At the end of 48 hours it is definitely enlarged, usually somewhat darker in color than normal; and close observation will show numerous very fine pin-point yellow areas. The organ enlarges rapidly so that at the end of 72 hours it is almost twice the normal size. The yellow areas enlarge to pin-head size and are thickly scattered over the entire surface of the organ. These areas are seen to be slightly depressed below the surrounding surface and apparently represent actual loss of liver substance. At the end of 4 days there is usually little change in the gross appearance of the organ except that the majority at this stage show evidences of biliary obstruction. They are usually dark green in color and quite swollen. From the 4th day to the 7th day, there is a diminution in the number of areas of yellow discoloration, although several large areas representing a confluence of smaller areas of necrosis persist. These areas gradually become quite pale in color. In those embryos which survive longer than 5 days there is no evidence of biliary obstruction, the liver returns to normal size and only a few large areas representing focal necrosis persist, the rest of the organ taking on a more normal appearance.

Microscopically at the end of 24 hours the liver shows cloudy swelling of all the parenchymal cells. The periportal vessels are dilated and filled with blood; the liver sinuses also appear to be more widely dilated than normally. Around the blood vessels are seen small accumulations of large mononuclears and here and there in the neighborhood of these cells, the parenchymal cells are undergoing degenerative changes. At 48 hours the microscopic lesion has advanced considerably. The entire organ is studded with focal areas of necrosis which are found adjacent to the larger blood vessels. There are numerous areas of extensive hemorrhage into the parenchymal tissue. The focal areas are characterized by a coagulative type of necrosis of the cytoplasm of the parenchymal cells; the nuclei are pyknotic and fragmented. Into these areas numerous large round mononuclears which have large round deep staining nuclei and a large amount of basophilic cytoplasm have accumulated. Polymorphonuclear leukocytes are exceedingly rare. At this time at the periphery of these necrotic foci within the cytoplasm of the more intact cells, inclusions resembling Guarnieri bodies are seen in large numbers. They range in size from very small granules to large triangular masses lying next to the nucleus. With hematoxylin and eosin they stain slightly basophilically and have a distinct purplish red cast. By 72 hours the

necrotizing process has advanced considerably, with only the centers of the lobules containing cells which appear fairly normal. Necrosis of the cells in the center of the foci is complete, only nuclear fragments and masses of coagulated cytoplasm being present. The infiltration of large mononuclears is much more abundant. Cytoplasmic inclusions in the cells at the periphery of these areas are still abundant. There is considerable free hemorrhage into the parenchymal tissues, the walls of the blood vessels are swollen, the endothelial lining is irregular in outline and the connective tissue cells of the vessel walls are fragmented. At this stage there is also marked necrosis of the epithelial cells of the biliary system; occasional cytoplasmic inclusions can be found, and accompanying this necrosis there is a beginning proliferation of these cells. Involvement of the Kupffer cells is not observed. At 96 hours there is a marked diminution in the size of the necrotic areas. Much of the coagulated cytoplasmic material and nuclear remains has disappeared and only a fine network of liver stroma is present. Around these areas there is often a zone of pale staining parenchymal cells which show only a cytoplasmic outline and a pale staining nucleus. Regeneration of the parenchymal cells is evidenced by numerous mitoses. Inclusions in the cytoplasm are very rarely seen. In most sections, at this stage, the striking characteristics are the large islands of rapidly proliferating epithelial cells of the bile ducts. They extend as large sheets and cords into the lobules. This evidently accounts for the evidences of biliary obstruction observed in the livers in the gross at this stage. From the 4th to the 7th day the process is one mainly of regeneration. The parenchymal cells become more normal in appearance and arrangement. The necrotic areas are replaced by normal tissues and only a few areas at the periphery of the organ remain in which evidences of a preexisting lesion are present. Here the foci of pale staining cells with large nuclei and distinct cytoplasmic outlines persist. There is a small amount of proliferation of connective tissue in these areas suggesting scar formation.

Spleen.—At the end of 24 hours after inoculation the spleen shows no abnormalities other than a slight swelling. By 48 hours there is marked enlargement and numerous small yellow foci are scattered over the entire surface of the organ. At 72 hours the yellow areas have greatly increased in size and appear as irregular nodules over the surface. The entire organ is at least twice the normal size. In several instances areas of hemorrhage are observed under the capsule. By the 4th day the swelling of the organ is subsiding; the focal necrotic areas are diminishing in size. Most of the spleens removed from embryos after 5 and 6 days appear quite normal except for a slight swelling. In a few spleens one or two large areas of focal necrosis persist.

Microscopically at the end of 24 hours the spleen shows very little evidence of infection. There is widening of the sinusoids and an occasional small collection of large mononuclears, around the vessels. Degeneration of small microscopic foci of cells evidenced by a granular pale staining cytoplasm and somewhat shrunken nuclei can be observed. At the 48 hour stage there is a diffuse involvement of the entire organ. There are numerous microscopic foci of necrosis. The

cellular cytoplasm is coagulated; the nuclei are pyknotic and fragmented. These areas are most advanced around the periphery of the blood vessels and directly beneath the capsule. In some of the more intact cells around the areas of necrosis cytoplasmic inclusions resembling Guarnieri bodies can be observed. There is a heavy infiltration of large mononuclears throughout the entire organ. Polymorphonuclear leukocytes are very rare. There are numerous areas of diffuse hemorrhages into the parenchyma. Edema of the organ is marked. The blood vessel walls are thickened and a heavy infiltration of mononuclears surrounds them. Where no necrosis is present, the cells all show some degenerative changes in that their cytoplasm is granular and pale staining; the nuclei are often irregular in outline. The process of necrosis reaches its height at the 72 hour stage. The focal necrotic areas are quite extensive, the entire organ containing numerous foci of coagulated cytoplasm and nuclear fragments. Cytoplasmic inclusions at this stage are very difficult to find. The mononuclear infiltration is quite extensive and occasional cells which have two or three nuclei with large cytoplasmic masses containing cellular debris are seen. After the 72 hour stage there is a gradual regeneration of the organ. The necrotic foci become smaller. Numerous mitotic figures suggest regeneration of the parenchymal cells. Beyond the 72 hour stage no evidence of cytoplasmic inclusions can be found. In some instances the process leaves a few areas in which the tissues are not regenerated and proliferation of fibrous tissue takes place. These are mostly found directly beneath the capsule.

Kidney.—In the gross the kidneys do not show anything remarkable during the entire course of the disease. In a few instances it is possible to note a slight amount of swelling but this is not definite enough to be of any particular significance.

Microscopically the kidney reveals the presence of very small areas of focal necrosis in the neighborhood of the blood vessels. These areas are very small and can only be detected with the oil immersion lens. The necrotic process most often affects the interstitial connective tissues and a few of the cells in the neighboring convoluted tubules. The cytoplasm of the cells is coagulated, the nuclei are pyknotic and there is a slight infiltration of large mononuclears. These areas are most common directly beneath the capsule and in the cortex. In a few of the cells, cytoplasmic inclusions resembling Guarnieri bodies are present. The glomeruli have not been observed to be affected by the disease process. The medullary portion does not seem to be affected. These changes are all observed between the 48 and 96 hour stage. Beyond this period the organ appears quite normal.

The adrenals in a few instances at the 72 hour stage show small necrotic foci in the cortex just beneath the capsule with round cell infiltration. Small microscopic areas in the medulla also show necrosis of the cells. No inclusions have been observed.

Heart.—In the gross the heart presents no observable changes during the course of the disease.

Microscopically during the 48 to 96 hour stage there are numerous small, focal

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have not been observed in the epithelial cells of the mucous layer. Focal collections of mononuclears with necrosis of the smooth muscle cells have also been frequently observed in the muscular and submucous layers of the small intestine in the earlier (48 to 96 hour) stages of the infection.

Pancreas.—Several sections of pancreas were obtained but no definite evidence of involvement of this organ has been observed.

Brain and Spinal Cord.—In the gross no evidence of involvement of these organs could be observed.

Numerous microscopic sections have shown no evidence of involvement of any of the nervous elements or supporting structure in either the brain, spinal cord or the spinal ganglia. Beginning with the 48 hour stage there are, however, numerous focal areas of mononuclear infiltration within the meninges especially at the base of the brain. These range in size from a collection of a few cells in the neighborhood of the blood vessels with subsequent necrosis of the connective tissue cells to large areas of infiltration affecting the meninges at the base of the brain. There is marked necrosis of the connective tissue cells. Numerous cytoplasmic inclusions can be observed in these cells during the 48 to 96 hour stages. At the 5th and 6th day involvement of the meninges has not been observed. No lesions have been observed in the meninges of the spinal cord.

Bone Marrow and Periosteum.—Numerous sections through the bones of the skull, ribs, vertebral column, femur and feet were made. It was observed that the same focal necrotic process affecting the other organs was also present in the bone marrow. These foci are most marked during the 48 to 96 hour stage of the infection. They are first observed as small perivascular collections of mononuclears. There is subsequent necrosis of the surrounding marrow cells so that by the 72 and 96 hour stage there are large areas of necrotic debris present throughout the marrow cavity. In the 48 to 72 hour stage cytoplasmic inclusions can be observed in the more intact cells. They are not found later than the 72 hour stage. Beyond the 96 hour stage the bone marrow gradually takes on a more normal appearance and by the 6th day no evidences of infection are present.

Throughout the entire course of the disease there is marked involvement of the periosteal tissues. As early as 24 hours, perivascular collections of large mononuclears can be found in numerous areas. These rapidly enlarge, necrosis of the connective tissue sets in and in many instances the process becomes so widespread that the entire periosteum of a femur becomes involved. There is here especially a very heavy infiltration of large mononuclears which causes an extreme thickening of the periosteum. The centers of these areas are made up of necrotic cells and cellular debris. In the 48 to 72 hour stages, numerous cytoplasmic inclusions are found in the connective tissue cells. In the stages beyond 96 hours, the necrotic areas disappear. An extreme thickening of the periosteum persists due to the proliferation in these areas of new connective tissue. This periosteal involvement is very striking and is present throughout the entire organism. Every section studied which contained bone structures showed evidence of involvement of the periosteum throughout the entire course of the disease.

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Throughout the entire course of the disease there is marked involvement of the periosteal tissues. As early as 24 hours, perivascular collections of large mononuclears can be found in numerous areas. These rapidly enlarge, necrosis of the connective tissue sets in and in many instances the process becomes so widespread that the entire periosteum of a femur becomes involved. There is here especially a very heavy infiltration of large mononuclears which causes an extreme thickening of the periosteum. The centers of these areas are made up of necrotic cells and cellular debris. In the 48 to 72 hour stages, numerous cytoplasmic inclusions are found in the connective tissue cells. In the stages beyond 96 hours, the necrotic areas disappear. An extreme thickening of the periosteum persists due to the proliferation in these areas of new connective tissue. This periosteal involvement is very striking and is present throughout the entire organism. Every section studied which contained bone structures showed evidence of involvement of the periosteum throughout the entire course of the disease.

have not been observed in the epithelial cells of the mucous layer. Focal collections of mononuclears with necrosis of the smooth muscle cells have also been frequently observed in the muscular and submucous layers of the small intestine in the earlier (48 to 96 hour) stages of the infection.

Pancreas.—Several sections of pancreas were obtained but no definite evidence of involvement of this organ has been observed.

Brain and Spinal Cord.—In the gross no evidence of involvement of these organs could be observed.

Numerous microscopic sections have shown no evidence of involvement of any of the nervous elements or supporting structure in either the brain, spinal cord or the spinal ganglia. Beginning with the 48 hour stage there are, however, numerous focal areas of mononuclear infiltration within the meninges especially at the base of the brain. These range in size from a collection of a few cells in the neighborhood of the blood vessels with subsequent necrosis of the connective tissue cells to large areas of infiltration affecting the meninges at the base of the brain. There is marked necrosis of the connective tissue cells. Numerous cytoplasmic inclusions can be observed in these cells during the 48 to 96 hour stages. At the 5th and 6th day involvement of the meninges has not been observed. No lesions have been observed in the meninges of the spinal cord.

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Membranal Lesion.—These lesions have been adequately described in other reports and will not be discussed in this paper as they do not belong to the embryo proper.

Presence of Paschen Corpuscles

Smears from the lesions of the different organs stained by the Morosow technique and Victoria blue after Herzberg (9) did not conclusively reveal the presence of Paschen bodies. There are too many other granules present in such preparations to make the detection of these bodies absolutely conclusive.

DISCUSSION

An analysis of the findings of these experiments leads to a few observations as to the behavior of vaccine virus when generalized throughout the organism. A local lesion is produced in the chorio-allantoic membrane. From this site the virus is disseminated, apparently by way of the blood stream, to various organs of the embryo. The rough quantitative estimation of the amount of virus in the various organs at the end of 72 hours shows that the virus is not equally distributed. The largest amount of the virus is present in the liver,

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spleen and bone marrow. Offhand, this would be expected since these organs are considered to have a distinct capacity for taking up foreign material from the blood stream. However, the histological study also shows that there are in the cells of these organs indications of a marked proliferation of the virus. These types of cells evidently offer a more suitable environment for the growth of the virus than do the cells of other organs, for example the central nervous system and the kidney.

In another report (10) we have described the special predilection of the dermal virus for the epithelial cells of the ectodermal layer in the membranal lesion. When the virus is disseminated from this focus by way of the blood stream it appears that throughout the organism the cells of mesodermal origin are highly susceptible to the action of the virus. The initial lesion is always found to be in and around the smaller vessels and capillaries. In this phenomenon it must not be overlooked that the mechanical effect produced by the slowing of the blood stream in these areas may be responsible, to a large extent, for the lodging of the virus in these areas.

It does not appear, however, that the vascular endothelium and perivascular structures are most favorable for the proliferation of the virus. The actual presence of inclusions, or widespread destruction of these tissues cannot be observed. The further proliferation of the virus depends upon the suitability of the cells in the environment of these initial foci. Thus in the liver, the bone marrow and the epithelial structures of the skin and mucosa a rich proliferation of the virus takes place in the cells of these organs. Tissues like the brain and glandular structures are evidently unsuited to the growth requirements of the virus.

It is also observed that a definite time element is indicated for the proliferation of the virus in the different tissue cells and the self limiting nature of the disease process is quite manifest. The presence of inclusion bodies within the cells may be taken as indicative of the proliferation of the virus. It appears to be of some significance that in all the organs where inclusion bodies are present they occur almost simultaneously and disappear rather suddenly from all the cells in which they are found after 96 hours following inoculation.

Pathologically the entire process is one of focal cellular proliferation and necrosis. The nature of the cellular reaction on the part of the

host to the virus of vaccinia is quite clearly shown. Since no other contaminating organisms are present to complicate the picture we have here a response to the pure virus infection. The reaction in the chick embryo is predominantly a mononuclear one and the impression is gained from our observations that these cells proliferate *in situ* and represent the mononuclears of the reticulo-endothelial system. These cells appear to be very active, and numerous mitotic figures are seen in them. Actual invasion of these cells by the virus indicated by inclusion bodies is not observed. Furthermore the absence of polymorphonuclear reaction is quite striking. This purely mononuclear response to a pure vaccinal infection has also been noted by Lillie and Armstrong (4).

The close analogy of generalized vaccinia produced in this manner to human variola is quite apparent. The lesions which have been described for the chick embryo closely correspond to those described by numerous students of human variola. The general distribution and the type of tissues affected are much the same as is also the focal character of the lesions.

In human variola the virus also apparently is disseminated from an original primary focus of infection, presumably the upper respiratory tract. While this analogy holds to some extent in the disease produced in the chick embryo it cannot be held to too strictly. Quantitatively the amount of inoculum used in this experiment is relatively much greater than may be presumed for the spontaneous disease in the human being. In the human being it is most likely that the dissemination of the virus depends entirely on its proliferation in its primary focus. In the chick embryo we cannot exclude the extreme likelihood of a sufficient quantity of virus reaching the blood stream direct from the inoculum very soon after inoculation. In this manner the almost simultaneous development of the disease process in the membrane and throughout the chick organism might be accounted for.

We have not, however, at any time observed in the vaccinal disease of the embryo, intranuclear inclusions described by some investigators in human variola.

We have, in this report, briefly described the salient features of the generalized disease produced in the chick embryo when inoculated by the chorio-allantoic route with a dermal strain of vaccine virus. A study of the more minute details of the pathogenesis of the disease

has not been undertaken and warrants further investigation by more refined methods of differential staining. The value of the chick embryo method of studying the pathogenesis of virus diseases is apparent and we present this work as a new approach to the study of virus diseases generally.

SUMMARY

1. Chick embryos infected by the chorio-allantoic route with a bacteria-free strain of vaccinia virus develop a general dissemination of the virus throughout the entire organism with the exception of the central nervous system.

2. Quantitative estimation of the distribution of the virus in the various organs of the infected chick by cutaneous inoculation on the rabbit skin offers no evidence for a heightened affinity of the virus for special tissues.

3. Histological study of the lesions in the various organs demonstrates the focal character of the lesions which apparently originate as perivascular infiltrations around the smaller blood vessels. No lesions could be demonstrated in the central nervous system proper.

4. In the earlier stages of the disease Guarnieri bodies are clearly demonstrable in the cells of the epidermis and the squamous epithelium of the buccal mucosa. Inclusion bodies closely resembling Guarnieri bodies are demonstrated in all the lesions occurring in the various other organs.

5. It was not possible to demonstrate conclusively the presence of Paschen bodies in the lesions of the internal organs by the Morosow method usually used for the demonstration of these bodies in the membranal lesion.

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EXPLANATION OF PLATES

PLATE 17

FIGS. 1 and 2. Photographs of a 20 day old chick embryo showing pustular eruption of the skin 7 days after inoculation with vaccinia virus on the chorio-allantoic membrane. Approximately actual size.

PLATE 18

FIG. 3. Photograph of the liver from a 17 day old chick embryo infected with vaccinia by inoculation of the chorio-allantoic membrane (72 hour infection). A normal liver from a chick embryo of the same age on the left.

FIG. 4. Section through a cutaneous vaccinal lesion in the chick embryo showing inclusion bodies in the epithelial cells (96 hour infection). Hematoxylin and eosin stain. × 1800.

FIG. 5. Section through the liver showing inclusion bodies in the liver cells (72 hour infection). Hematoxylin and eosin stain. × 1800.

FIG. 6. Section through a lesion in the skin showing mononuclear infiltration in the corium and proliferation of the overlying epithelial cells (96 hour lesion). Hematoxylin and eosin stain. × 100.

FIG. 7. Section through a lesion in the skin showing proliferation of epithelial cells and necrosis in the center (5 day lesions). Hematoxylin and eosin stain. × 150.

FIG. 8. Section through a pustule in the skin showing liquefaction of necrotic cells in the center and hyperplasia of the epithelial cells at the edge of the lesion (7 day lesion). Hematoxylin and eosin stain. × 100.

PLATE 19

FIG. 9. Section through the brain and meninges showing involvement of the meninges by mononuclear infiltration (96 hour lesion). Hematoxylin and eosin stain. × 150.

FIG. 10. Section through the knee joint showing involvement of the synovial membranes (96 hour lesion). Hematoxylin and eosin stain. × 150.

FIG. 11. Section through the stomach wall showing a focal area of mononuclear infiltration under the visceral peritoneum (72 hour lesion). Hematoxylin and eosin stain. × 100.

FIG. 12. Section through the stomach showing an area of focal necrosis in the smooth muscle layer (96 hour lesion). Hematoxylin and eosin stain. × 250.

FIG. 13. Section through the bone marrow showing areas of focal necrosis (96 hour lesion). Hematoxylin and eosin stain. × 250.

FIG. 14. Section through the liver showing hyperplasia of the bile duct epithelium (96 hour lesion). Hematoxylin and eosin stain. × 250.

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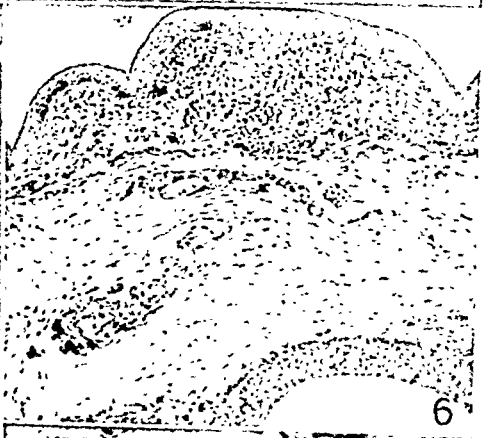

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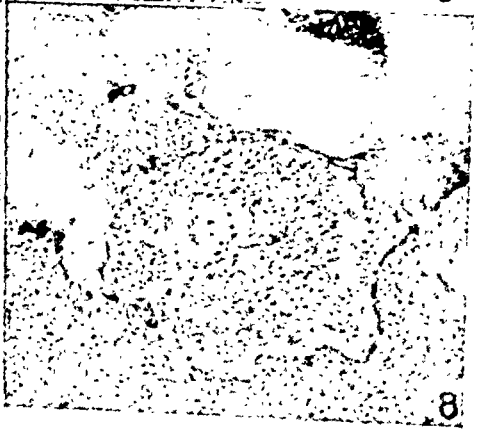

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STUDIES ON THE ETIOLOGY OF RABBIT POX

I. Isolation of a Filterable Agent: Its Pathogenic Properties

By LOUISE PEARCE, M.D., PAUL D. ROSAHN, M.D., and CH'UAN-K'UEI HU, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

Plate 20

(Received for publication, September 26, 1935)

The investigations reported in this and succeeding papers (1–4) had their inception in a highly contagious and fatal epidemic which broke out with explosive violence in a rabbit breeding colony at The Rockefeller Institute in December, 1932. Within a month practically every animal in the colony of over 1,400 rabbits had been infected. The disease resembled small pox of man in so many respects that it was called rabbit pox. Its clinical and pathological aspects have been described by Greene (5, 6). Suffice it to say here that it was characterized by a pock-like eruption in the skin and mucous membranes, lymphadenitis, eye involvement, coryza, an orchitis in male animals, and various constitutional symptoms including fever. The incubation period varied from 5 to 14 days. The course was variable. Death frequently occurred within a few hours after the first signs of the infection were noted or after a period of a few days or weeks. On the other hand, recovery from a severe infection took place in many instances, visible lesions of the skin frequently healing with scar formation. Mild and asymptomatic cases were also seen. Although the mortality rate was variable, the disease was extremely destructive and 46 per cent of the colony succumbed. The rate was highest in young animals 4 to 8 weeks old, while in those less than 14 weeks of age it was 72 per cent. In adults the mean mortality was about 15 per cent, but the rate in different breeds varied widely. The infection produced the severest symptoms in pregnant and lactating does, and pregnancy was commonly terminated by abortion. During the termi-

nal stages of the epidemic, 6 to 8 weeks after its onset, mild and abortive types of infection were common and a large proportion of animals of the most susceptible breeds and age groups survived infection contracted at that time.

The literature contains little information which throws any light on the situation with which we were confronted. Mahlich (7) in a handbook for rabbit breeders and fanciers published in 1919 mentions a disease *Pocken* which "fortunately occurs very rarely in our rabbit population." The brief description of the generalized cutaneous eruption, together with the statement that the best remedy is killing affected animals, strongly suggests that the disease is the same as or very like the epidemic disease which we encountered.

The pock-like character of the cutaneous eruption in the epidemic disease in our colony was extremely striking and on this basis alone, the condition would unquestionably be classified in the pock group of diseases. The question naturally arose as to whether vaccine virus was concerned. At the time of the outbreak, however, there was no evident source of vaccine virus. Furthermore, vaccinal infection arising from exposure or contact is generally considered to be a mild or asymptomatic condition although there have been instances of severe epidemics which have been ascribed to neurovaccine virus. Investigations of these epidemics, however, particularly from the standpoint of comprehensive experiments to determine the precise nature of the etiological agents were apparently not carried out. It was subsequently learned that in at least three other laboratories of the Institute rabbits had been inoculated with vaccine virus prior to the epidemic and that in two of these neurovaccine had been used. The possibility of conveyance of a highly contagious infection from one group of animals to another must be considered, but if a spread of vaccinia among the experimental animals in these other laboratories took place, it apparently was not detected before the outbreak of the present epidemic in the breeding colony. In this connection, it should be mentioned that it was not the first time we have encountered this disease. In the spring of 1930 there was a small and much less severe epidemic of a disease presenting the same clinical and pathological picture. Its effects were comparatively mild and with the recovery or elimination of affected animals, fresh cases ceased to develop.

Early in the course of the epidemic, attempts to transmit the infec-

tion experimentally were made for the purpose of identification of the causative agent with the hope that means might be found to prevent future outbreaks. Experimental transmission of the infection was readily accomplished by rabbit inoculation of Berkefeld V filtrates or of unfiltered emulsions of tissues derived from spontaneous cases. The causative agent was easily filterable through Berkefeld V candles and was transmitted by tissue filtrates for 15 consecutive rabbit passages. The characteristic features of the virus persisted throughout the 9 months from January to October, 1933, in which the experimental investigations were carried out. The results of various tests showed that although the virus was related to vaccine virus, it was not completely identical with two specimens of dermovaccine and one of neurovaccine used for comparison. The results of the experiments which demonstrated this relationship led to the opinion that vaccination with dermovaccine might constitute an efficient prophylaxis against the spontaneous disease.

The results of the investigation on the filterable agent and the experimental infection which have been referred to in various preliminary communications (8) are now reported in detail. The study is obviously incomplete, but after information on certain major points had been obtained it was decided that further work should not be undertaken because at this time the necessary isolation facilities were not available. Many phases of the problem as for example, a study of the filtration properties of the virus, particularly from the standpoint of comparisons with other viruses, were only touched upon or were omitted altogether. Furthermore, certain aspects of the work, such as complete histological examinations of post mortem material had to be omitted because of the demands of other experiments. In the present paper the experiments dealing with the isolation, serial passage, and certain pathogenic properties of the virus are reported, together with a description of the acute fulminating and rapidly fatal type of infection associated with the routine serial passage of the virus. Subsequent papers deal with the clinical manifestations and course of the less acute type of the experimentally induced disease which was indistinguishable from the spontaneous condition (1); with the results of various experiments, chiefly of an immunological character, in which the virus was compared with other filterable

viruses (2, 3); and finally, with experiments in which the susceptibility of certain other animal species to inoculation of the virus was studied (4). The last paper also contains a general discussion of the work on experimental pox with special reference to the nature of the virus, and the significance of rabbit pox to other pock diseases.

Materials and Methods

The successful experimental transmission of rabbit pox was made from spontaneous cases by the injection of both filtered and unfiltered emulsions of tissues into the testicles of normal rabbits. 4 strains of the virus were thus obtained. For a short period, the strains were carried by serial rabbit transfer, but as soon as it was certain that there was no difficulty in preserving an active agent by rabbit passage, only 1 strain (Xy171) was retained for routine work. Later in the course of the epidemic, additional specimens of virus were recovered from other spontaneous cases.

Organ Source of Virus.—The agent was recovered from the following organs and tissues of cases of the spontaneous disease: testicle, ovary, liver, spleen, popliteal and inguinal lymph nodes, brain, spinal cord, whole blood, defibrinated blood, and skin.

Animals.—Male rabbits in most instances obtained from dealers were used for inoculation. There was no evidence at any time that they had been affected by pox. They were approximately 4 to 6 months old and were of the usual hybrid type, the common greys, browns, Flemish crosses, and Chinchillas predominating. In some experiments younger rabbits were used.

Routines of Inoculation.—The initial injections were made intratesticularly and this method was followed for the routine passage of virus. Other routes employed in various experiments were: intravenous, intracutaneous, subcutaneous, intramuscular, intraperitoneal, intracerebral, nasal, and conjunctival instillation, and the application of tissue-virus emulsions to scarified areas of skin and cornea. Exposure in the rooms and in cages in which experimental animals were kept was also made.

Dosage.—In most experiments an involved testicle was used for inoculation and both filtered and unfiltered emulsions were employed. Emulsions were prepared in the usual manner with Locke's solution by grinding with alundum and a 10 or 15 per cent suspension by weight was ordinarily made. For the routine passage of virus, rabbits were injected in one or both testicles and in most instances with a dose of 0.5 cc. With other routes various dilutions of tissue-virus suspension as well as variable amounts were injected; these will be mentioned in connection with the particular experiments.

Filtration.—The virus in a Locke solution tissue emulsion was easily filtered through Berkefeld V candles. In certain experiments Berkefeld N and W candles and Seitz filters were used. All filtrations were made under negative pressure (air vacuum) varying from 100 to 500 mm. of Hg in from 2 to 8 minutes. The

procedure was controlled by adding the saline washing from a 24 hour agar slant culture of *B. prodigiosus* to the tissue emulsion before filtering in an amount equivalent to 10 per cent of the emulsion.

Examination of Tissues.—The majority of autopsies were performed immediately after the animals had died or after they had been sacrificed by means of an injection of air into a marginal ear vein. Aerobic and anaerobic cultures in broth and other media for the detection of ordinary bacteria were made of tissues used for inoculation and in many instances from other organs as well. Tissues were fixed in Zenker's fluid, Helley's fluid, and 10 per cent formalin, and sections were stained with hematoxylin and eosin and with Giemsa's stain.

Recovery of Virus from Spontaneous Cases

The results of bacteriological examinations of organs and tissues from spontaneous cases revealed no organism with which the disease could be induced. The intratesticular injection of Berkefeld V[1] filtrates from a variety of such tissues, however, produced an acute reaction in rabbits which was characterized by fever, a marked hemorrhagic orchitis, and death. Furthermore, this reaction was regularly reproduced in a consecutive series of rabbit to rabbit passages by means of Berkefeld V filtrates of tissue emulsions as well as by unfiltered emulsions. The less acute type of reaction also induced by Berkefeld V filtrates was indistinguishable from spontaneous cases of pox. This type of disease was associated particularly with routes of injection other than the intratesticular and with a smaller dosage as is described in the following paper (1). In all cases the filtrates were cultured aerobically and anaerobically in broth. In the following discussion all references to filtrates used are understood to indicate sterile filtrates.

The spontaneous cases from which tissues were obtained for inoculation will first be briefly described.

All inoculations were made intratesticularly. To avoid repetition, the results are simply stated as "positive" or "successful," "negative" or "unsuccessful." The positive results refer to the development of an acute orchitis and fever and a fatal outcome. These features are fully discussed in the following section in which the results of the serial passage of the virus are taken up. In the absence of these clinical features, the results of inoculation were considered to be negative.

[1] Experiments with other filters were limited to a few preliminary tests. The results indicated that the virus was filterable through Berkefeld N and Seitz filters. In the case of Berkefeld W candles the results were uncertain.

Rabbit Xy171.—Sable Marten hybrid male, 8 months old. Well marked acute symptoms including diffuse orchitis, popliteal adenitis, mucopurulent nasal and conjunctival discharge, slight papular cutaneous eruption over body and ears; general weakness and listlessness. Killed. 0.5 cc. of the Berkefeld V filtrate of the right testicle injected in each testicle of 3 rabbits and 1 cc. in one testicle of a 4th rabbit; in 2 of these animals 0.5 cc. was dropped in the right nostril. 0.5 cc. of the Berkefeld V filtrate of defibrinated heart's blood was injected in each testicle of 2 rabbits and 0.5 cc. was injected into one testicle of a 3rd rabbit. All 4 rabbits injected with testicular emulsion and 1 rabbit injected with defibrinated blood were successfully inoculated.

The majority of experiments were carried out with the virus obtained from an animal inoculated with testicular emulsion.

Rabbit 782.—Himalayan hybrid breeding doe 11 months old. Marked weakness and prostration of a few days' duration, profuse blood stained mucopurulent discharge, mucoserous conjunctival discharge, mucous anal discharge, diarrhea, sparse cutaneous maculopapular eruption on ears and body, emaciation. Found dead. 0.5 cc. of the Berkefeld V filtrate of an emulsion of liver, spleen, and ovaries was injected in both testicles of 2 rabbits with positive results.

Rabbit AB18.—American Blue doe 9 weeks old. Very recent widespread papular eruption on ears, eyelids, nose, lips, genital region, and body; pronounced cutaneous edema of the hind feet; enlargement and induration of the superficial lymph nodes, especially of the popliteals; no nasal or conjunctival discharge. Killed. 2 rabbits were injected with doses of 0.5 cc. of an unfiltered suspension of popliteal lymph nodes; 2 rabbits were injected with 1.0 cc. doses of an unfiltered liver and spleen emulsion; and 2 rabbits received 1.0 cc. of whole blood. All 6 rabbits were successfully inoculated.

Rabbit P2.—Polish doe 14 months old. Fulminating infection of less than a week's duration. Marked ophthalmia with conjunctival serous discharge, watery nasal discharge, marked respiratory distress, pronounced edema of anogenital region, and enlarged, tense popliteal lymph nodes; muscular tremors, incoordination, a tendency toward convulsions, and almost complete paralysis of legs with twisting of body. Died. Unfiltered suspensions of the following organs were injected in both testicles of each of 2 rabbits: brain (0.5 and 0.2 cc.); spinal cord (0.5 and 0.1 cc.); and 1 rabbit each with liver (0.5 cc.) and spleen (0.5 cc.). All 6 rabbits were successfully inoculated. A rabbit injected with lung filtrate showed no clinical evidence of infection.

Rabbit X667-1.—Hybrid male, 5 weeks old. Early very severe case occurring toward the end of the epidemic. Extremely profuse papular skin eruption on the body, extremities, head and ears, serous nasal discharge with bloody crusts and papules about nares and lips. Killed. Liver filtrate in 0.1 and 0.03 cc. doses was injected in one testicle of each of 2 rabbits with negative clinical results. The injection of a skin filtrate (0.5 and 0.2 cc.) in one testicle of each of 2 rabbits gave positive results in both cases.

Rabbit HA46-1.—Havana male, 2 months old. Recent mild case developing at the end of the epidemic. There was a small cutaneous papule in one ear and two larger ones on the back; no constitutional symptoms. Killed. Unfiltered emul-

sions of the testicle, liver, and popliteal and right inguinal lymph nodes were injected. The results on the 2 rabbits injected with lymph node and testicle suspensions respectively were positive, but that on the rabbit injected with liver emulsion was negative.

Rabbit 8415.—Hybrid adult male, approximately 8 months old. A recovered case of about a week's duration. The condition which had included an orchitis had been mild. Killed. Negative post mortem findings. Unfiltered and filtered suspensions of the right testicle and of both popliteal lymph nodes were injected in 0.5 cc. doses in both testicles of 4 rabbits. A positive result was obtained in the rabbit injected with unfiltered testicular emulsion, but no clinical signs developed in the other animals.

Rabbit X213-1.—Hybrid male, 8 months old. Apparent complete recovery of approximately 3 weeks' duration; disease had been moderately severe with a diffuse orchitis, a few skin papules, marked right ophthalmia and keratitis, and moderate popliteal adenitis. The right testicle and right popliteal lymph node were removed under ether anesthesia; both organs appeared normal. Unfiltered and filtered testicular suspensions and an unfiltered emulsion of the popliteal lymph node were injected intratesticularly in 4 rabbits. The equivocal results which were obtained were regarded as negative.

Summary of Results of Original Inoculations from Spontaneous Pox Cases.—The intratesticular injection in rabbits of tissues from 6 cases of spontaneous pox of varying severity and duration produced a pronounced local and general reaction which, as will be described in the following section, could be duplicated by the serial passage of affected tissue. The marked acute hemorrhagic orchitis was especially striking. Positive results were obtained from the inoculation of Berkefeld V filtrates of tissues from 3 cases and with unfiltered tissue emulsions from 3 other cases.

In 5 of these cases the disease was well marked or pronounced while in the 6th it was very mild. Positive results were obtained in 23 of the 29 rabbits injected, or 79 per cent. The tissues and organs with which these findings were obtained were the following: testicle, ovary, liver, spleen, brain, spinal cord, popliteal lymph nodes, skin, whole and defibrinated blood. The failure to induce clinically positive results occurred in the case of certain materials obtained from 4 of the 6 rabbits, that is, in 2 rabbits injected with filtered defibrinated blood, in 2 injected with filtered liver emulsion, in 1 injected with unfiltered liver emulsion, and in 1 rabbit injected with filtered lung suspension. Among 14 rabbits injected with Berkefeld V filtrates, positive clinical results developed in 9 or 64 per cent, and among 15 rabbits injected with unfiltered materials in 14 or 93 per cent. The single failure encountered with the use of unfiltered material was with a suspension of liver obtained from a mild case of the disease.

From 1 of 2 rabbits which had recovered for about a week from a moderately severe attack, the virus was demonstrated in the unfiltered but not in the filtered

testicular emulsion; it was not demonstrated in either the filtered or the unfiltered suspension of the popliteal lymph nodes. From the 2nd rabbit with a recovery period of approximately 3 weeks after a comparatively severe attack, the right testicle and right popliteal lymph node were used; the injection of 4 rabbits with filtered and unfiltered emulsions gave negative clinical results.

Serial Passage of Virus. Acute Fulminating Infection

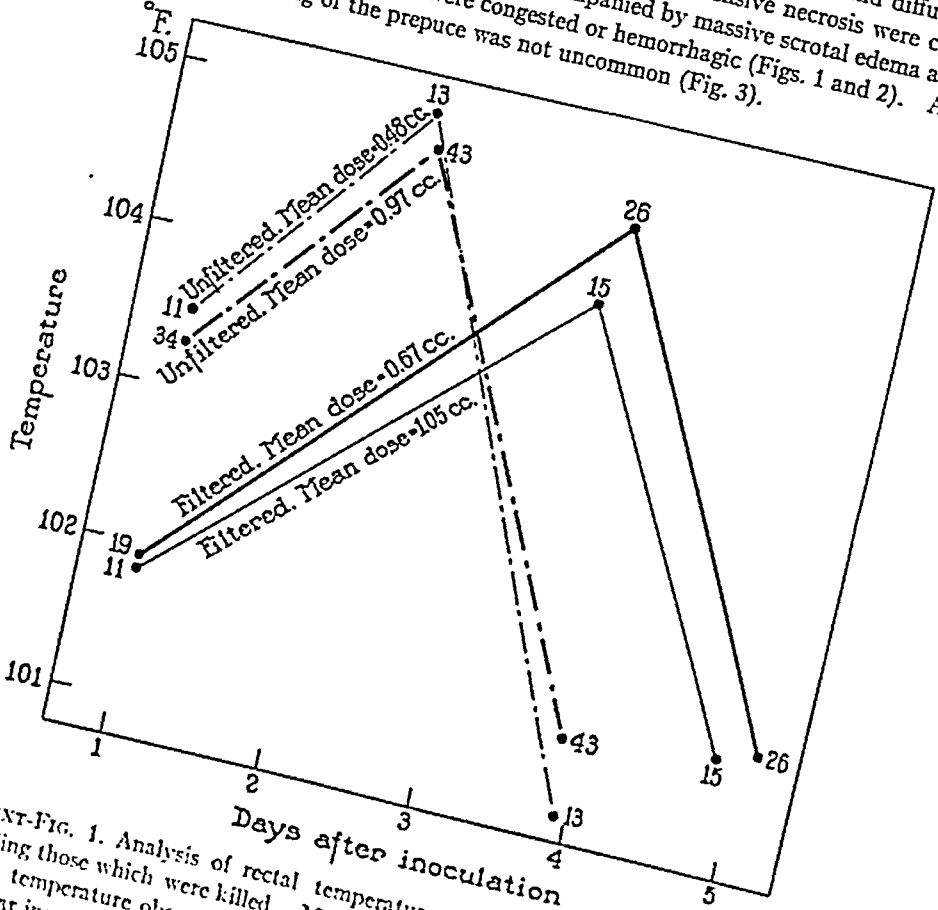
Two clinical types of the experimental disease predominated. Under conditions of the routine serial passage of virus in which filtered or unfiltered testicular tissue suspensions were injected by the intratesticular route, the infection was of an acute fulminating nature and with few exceptions resulted fatally within a week and usually within 3 to 5 days. In these cases the clinical manifestations were largely confined to the testicular lesion and to fever. In circumstances which permitted a longer survival or recovery, a wide variety of symptoms characteristic of spontaneous pox developed (1). These circumstances included the use of a very small dosage in the case of testicular tissue-virus injected intratesticularly, the employment of routes of injection other than the intratesticular, and finally, the use of tissues other than the testicle as a source of virus.

The Xy171 strain of virus which was used for most experiments was carried for 15 consecutive passages by means of bacteriologically sterile Berkefeld V filtrates of fresh or 24 hour old testicular emulsions kept at ice box temperature. Injections were made in one or both testicles of rabbits, usually in 0.5 cc. amounts. Beginning with the 9th generation and continuing for 10 serial passages, unfiltered testicular emulsions were also used. The results of the first passage from the spontaneous case (rabbit Xy171[2]) did not differ from those observed in subsequent generations, and with two exceptions to be discussed later, a level of high virulence was continuously maintained in both the filtered and the unfiltered series. The only essential difference in the reaction to the filtered as compared with the unfiltered inoculum was that of time, that is, the initiation and duration of the reaction and the fatal outcome occurred 1 to 3 days later in the case of rabbits inoculated with filtrates. The explanation of this difference is presumably that of a lowered virus content brought about by the process of filtration.

The three outstanding features of the acute fulminating type of reaction were a hemorrhagic orchitis with an accompanying scrotal edema, fever, and death.

[2] The positive results obtained with tissues from other spontaneous cases listed in the previous section were entirely similar to those obtained from rabbit Xy171. These other strains, however, were not carried for as many consecutive animal passages.

Orchitis.—The local reaction in the inoculated testicle was extremely marked. Usually within 24 hours small areas of induration or a diffuse resistance were palpable. By the 2nd or 3rd day, the testicle was greatly swollen and diffusely indurated and marked congestion, hemorrhage, and extensive necrosis were conspicuous features. The condition was accompanied by massive scrotal edema and frequently, the scrotal tissues were congested or hemorrhagic (Figs. 1 and 2). An edematous swelling of the prepuce was not uncommon (Fig. 3).



TEXT-FIG. 1. Analysis of rectal temperatures of acute rabbit pox animals, excluding those which were killed. Mean temperature 24 hours after inoculation, highest temperature observed, and last temperature taken before death. Intratesticular inoculations of unfiltered and Berkefeld V filtered testicular tissue-virus emulsions.

Fever.—Fever regularly developed and was usually present on the 2nd or 3rd day, rectal temperatures of 105° and 106°F. were frequent. Some time before death, usually within 24 or 48 hours, an abrupt fall in the temperature to subnormal levels was often observed. In other instances, the last temperature recorded was abnormally high.

testicular emulsion; it was not demonstrated in either the filtered or the unfiltered suspension of the popliteal lymph nodes. From the 2nd rabbit with a recovery period of approximately 3 weeks after a comparatively severe attack, the right testicle and right popliteal lymph node were used; the injection of 4 rabbits with filtered and unfiltered emulsions gave negative clinical results.

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26 or 84 per cent, occurred on the 5th, 6th, and 7th days after inoculation, and that none occurred as early as the 3rd day. In contrast to this finding 6 or 8 per cent of the rabbits injected with unfiltered emulsions died on the 3rd day and the remainder on the 4th and 5th days.

The question of dosage in these two groups of rabbits is not considered in the above tabulation because it was found that within wide limits of dosage, comparable results were obtained. Thus, of the 31 rabbits injected with filtrates, 20 which received doses of 0.1 to 1.0 cc. were found dead in 4 to 8 days or on an average of 5.8 days. 6 rabbits given 2.0 or 3.0 cc. were found dead in 5, 6, or 7 days, or on an average of 6 days. And 5 rabbits injected with amounts of 0.0016 to 0.05 cc. were found dead in 4 to 8 days, or on an average of 6 days. The dosage of filtrates injected was more variable than that of unfiltered emulsions because most of the work with filtrates was done early in the course of the investigation when the question of dosage was being studied. Of the 39 rabbits injected with unfiltered inocula, 27 which received doses of 1.0 cc. were found dead in 3 to 5 days, or on an average of 4.1 days. 3 rabbits given doses of 2.0 cc. were found dead in 2, 3, and 5 days, or on an average of 3.3 days. 6 animals injected with 0.6 cc. doses were found dead in 3 to 5 days, or on an average of 4.2 days. 2 others given 0.4 cc. doses and 1 given 0.2 cc. were found dead in 4 and 5 days.

General Symptomatology.—In addition to the three principal features of the acute fulminating condition, there were others of a more general nature. The animals became obviously ill. Apathy and a disinclination to move were commonly seen, the fur was dull and rough, the appetite was diminished, the animal often became thin and weak, and frequently a mucous diarrhea developed (Fig. 3). Prostration of varying degree was usually seen a day or two before death. In other cases, however, there was a remarkable absence of general symptoms of illness, and the rabbit would appear to be in good condition as late as the day before death.

In animals surviving for as long as a week, a maculopapular eruption of the skin and mucocutaneous borders, a blepharitis and conjunctivitis, and a watery nasal discharge were sometimes observed. These manifestations were characteristic features of the less acute experimental infection which is discussed in the following paper (1), and consequently need only to be mentioned here.

Variations of the Acute Fulminating Infection

A fatal outcome occurred so regularly in rabbits injected intratesticularly with testicular tissue-virus emulsion even under conditions

The curves in Text-fig. 1 illustrate the character of the febrile reaction. The curves were drawn from three mean temperature values, namely, 24 hours after inoculation, the highest temperature recorded and the last temperature taken within 24 hours of death. Both filtered and unfiltered inocula are represented, but the values for all animals which were killed were excluded. It will be noted that in the case of the group injected with unfiltered emulsions the temperature reached febrile levels within the first day after inoculation, whereas the mean value for the filtrate series was still within normal limits. The highest values recorded occurred between the 2nd and 3rd day after inoculation in the case of the unfiltered group and between the 3rd and 4th day in the filtrate group. A similar time difference is seen in the values for the last temperature within 24 hours of death. Furthermore, the mean value of the highest temperatures recorded for the unfiltered series exceeded that for the animals injected with filtrates.

The mean amount of filtrate used for inoculation was 0.67 cc. and of unfiltered emulsion 0.97 cc. That the difference in the febrile reaction of the two groups was not attributable to the differences in volume of inocula but was a matter of virus content is shown by the second set of curves, the values for which were obtained by omitting the temperature records on all animals inoculated with doses of more than 2.0 and less than 0.6 cc. in the case of filtrates and of more than 0.9 cc. in the case of unfiltered material. In this calculation the mean dosage for the filtrate group, 1.05 cc., was more than twice the size of that for the unfiltered group, 0.48 cc. The second curves differ in no essential respect from the first ones.

Fatal Outcome.—The third feature of the reaction in the acute fulminating type of experimental pox was a fatal outcome. This result is shown in the following tabulation which summarizes the observations on 70 rabbits inoculated with the Xy171 strain in which the disease was allowed to progress uninterruptedly. Of these animals 49 belonged to the routine passage series and 21 were inoculated for other purposes. 5 passage animals have been omitted because of recovery or an unusually long survival; these exceptional findings are discussed in the following section.

Inoculum	No. of rabbits	No. of deaths	Mean time of death
		per cent	*days*
Filtered testicular tissue emulsion	31	100	5.84
Unfiltered " " "	39	100	4.1

		Day of death							
		1	2	3	4	5	6	7	8
Filtered testicular tissue emulsion	31	—	—	—	3	8	13	5	2
Unfiltered " " "	39	—	—	6	23	10	—	—	—

These observations show that in the case of intratesticular injection of Berkefeld V filtrates of testicular tissue-virus, the majority of deaths,

26 or 84 per cent, occurred on the 5th, 6th, and 7th days after inoculation, and that none occurred as early as the 3rd day. In contrast to this finding 6 or 8 per cent of the rabbits injected with unfiltered emulsions died on the 3rd day and the remainder on the 4th and 5th days.

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In animals surviving for as long as a week, a maculopapular eruption of the skin and mucocutaneous borders, a blepharitis and conjunctivitis, and a watery nasal discharge were sometimes observed. These manifestations were characteristic features of the less acute experimental infection which is discussed in the following paper (1), and consequently need only to be mentioned here.

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A fatal outcome occurred so regularly in rabbits injected intratesticularly with testicular tissue-virus emulsion even under conditions

of a small dosage that the few exceptions to the general rule merit special mention.

The true incidence of recovery cannot be determined because the disease was not allowed to progress to an uninterrupted conclusion in all cases. Stock animals were not infrequently sacrificed to furnish material for the next passage of virus and for other experiments in order to avoid bacterial contamination of tissues. They were killed on an average of a day before the mean time of death of the other animals. For example, in the routine filtrate series of the Xy171 strain with which most of the experimental work was done, 10 of the rabbits were killed at a mean time of 5 days after inoculation. It will be recalled that the mean time of death of 31 rabbits with an uninterrupted disease was 5.8 days. In the case of unfiltered inocula, 6 rabbits were killed on an average of 3.3 days after inoculation and the mean time of death of 39 animals was 4.1 days. While it cannot be said that none of these 16 rabbits might not have recovered, there is a reasonable certainty that at least the majority would have died. But among the serial passage rabbits of the Xy171 strain of virus which were not sacrificed, there were 2 instances of recovery, the explanation of which is not apparent.

In the 8th generation of the filtrate series, 3 rabbits inoculated in one testicle with 0.5, 0.25, and 0.1 cc. respectively developed typical local and febrile reactions. The 2 rabbits given the largest doses were killed on the 5th day and material from them was used to inoculate the 9th generation rabbits. The 3rd animal which was castrated on the 9th day developed a scanty papular cutaneous eruption and eventually recovered. Meanwhile, of the 9th generation 2 animals inoculated with 1.0 and 0.5 cc. of filtered virus emulsion developed a less pronounced testicular reaction than usual, the development of fever was delayed, and death did not occur until the 10th day. The 3rd rabbit of the 9th generation was inoculated with bilateral testicular injections of 0.2 cc. and 5 intracutaneous injections of dilutions of 1:10 to 1:100,000. No generalized clinical manifestations were observed except a diarrhea and death was delayed to the 18th day. In both the 8th and 9th rabbit passages, the disease was thus definitely less severe than in the previous generations. In the next 4 serial passages, the larger doses of 2 and 3 cc. were injected; these rabbits died on the 5th, 6th, and 7th days. The 15th generation comprised 2 rabbits inoculated with 2.0 and 1.0 cc. doses respectively. These animals were found dead on the 5th and 6th days. Although the numbers of animals are small and the doses employed were larger than those ordinarily used, the resumption of a fatal outcome within the time previously experienced indicates that the severity of the infection had approached or had resumed its former high level.

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With the AB18 strain of virus[3] there were 4 instances of recovery after the intratesticular injection of testicular tissue emulsions which had been filtered twice through Berkefeld V candles before a sterile filtrate was obtained. 3 of the cases were observed in a group of 5 rabbits which were inoculated in one testicle with 0.1 cc. doses. The first rabbit injected with full strength filtrate recovered after the development of a typical local and febrile reaction, a metastatic orchitis, typical papular lesions of the lips and nose, and involvement of the eyes. The other rabbits were injected with dilutions of 1:10, 1:100, 1:1,000, and 1:10,000 respectively. All developed well marked testicular and febrile reactions; the 2 injected with the lower dilutions died on the 7th and 13th days while those which received the higher dilutions recovered. The filtrate was kept in the refrigerator and 7 days after the first inoculation, a rabbit was injected in one testicle with 0.1 cc. and 6 days later, or 13 days after the first inoculation, 2 rabbits were injected in one testicle with 0.5 and 0.1 cc. respectively. Full strength filtrate was used for these injections. All 3 rabbits developed fever and a typical orchitis. The first was ill and was killed on the 8th day; the others were found dead on the 9th day. The 4th instance of recovery was a rabbit inoculated in one testicle with 0.1 cc. of a testicular tissue-virus emulsion which had been filtered through 2 Berkefeld V candles. This animal developed typical local and generalized manifestations of pox and was severely ill, but recovery eventually took place.

As far as the results on these 4 animals inoculated with the AB18 strain of virus are concerned, it appears that a sufficiently small dosage was obtained by means of a double filtration and in 2 cases with the added factor of virus dilution, so that the usual fatal outcome was avoided although it was potent enough to produce unmistakable clinical symptoms. But this explanation cannot be applied to the 2 instances of recovery in the Xy171 strain for dilution of virus was not made. No reason for this unusual result could be found at the time. An explanation based on individual animal variation with increased resistance does not seem applicable to the results observed in connection with the first of these cases. It is possible that elimination of virus brought about by castration on the 9th day was sufficient to turn the tide toward recovery in an animal that had already survived

[3] There was no indication that the AB18 strain of virus differed from the Xy171 strain unless the present instances of recovery be so considered. Fewer experiments were done with the strain so that the incidence of recoveries was relatively much higher. In the absence of a sufficient number of comparable tests, however, the difference in results cannot be ascribed to an essential difference in the strains.

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The only other instance of recovery after intratesticular injection of the Xy171 strain of virus in testicular tissue occurred after the inoculation of unfiltered material. 2 rabbits injected in both testicles with 0.5 cc. doses developed a typical local and febrile reaction. 1 animal died on the 5th day; the other developed a generalized cutaneous papular eruption and recovered. 2 other rabbits inocu-

lated intradermally with the same specimen of virus died on the 8th and 10th days respectively.

With the AB18 strain of virus[3] there were 4 instances of recovery after the intratesticular injection of testicular tissue emulsions which had been filtered twice through Berkefeld V candles before a sterile filtrate was obtained. 3 of the cases were observed in a group of 5 rabbits which were inoculated in one testicle with 0.1 cc. doses. The first rabbit injected with full strength filtrate recovered after the development of a typical local and febrile reaction, a metastatic orchitis, typical papular lesions of the lips and nose, and involvement of the eyes. The other rabbits were injected with dilutions of 1:10, 1:100, 1:1,000, and 1:10,000 respectively. All developed well marked testicular and febrile reactions; the 2 injected with the lower dilutions died on the 7th and 13th days while those which received the higher dilutions recovered. The filtrate was kept in the refrigerator and 7 days after the first inoculation, a rabbit was injected in one testicle with 0.1 cc. and 6 days later, or 13 days after the first inoculation, 2 rabbits were injected in one testicle with 0.5 and 0.1 cc. respectively. Full strength filtrate was used for these injections. All 3 rabbits developed fever and a typical orchitis. The first was ill and was killed on the 8th day; the others were found dead on the 9th day. The 4th instance of recovery was a rabbit inoculated in one testicle with 0.1 cc. of a testicular tissue-virus emulsion which had been filtered through 2 Berkefeld V candles. This animal developed typical local and generalized manifestations of pox and was severely ill, but recovery eventually took place.

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longer than the average time. On the other hand, the decreased severity of the infection was continued over to the next passage animals which were not inoculated with material from the rabbit which eventually recovered, and this finding was in turn followed by an apparent resumption of disease severity. That these results may have been brought about by changes in virulence of the virus should be considered, but no evidence for such an explanation is apparent.

Retention of Virulence of Virus in Stored Tissue

The virulence of the virus was retained under conditions of the storage of tissues in the refrigerator, either with or without the addition of 50 per cent glycerol for as long as 127 days, as shown by the results obtained in 14 experiments, 12 with the Xy171 strain and 1 each with the AB18 and X667 strains. A total of 28 rabbits were injected with these tissues.

Six experiments were carried out with Berkefeld V filtrates of tissues stored in the ice box for 13 to 67 days (Table I). In Experiments 2, 3, 4, and 5 the emulsions for injection were prepared from testicular tissue preserved in 50 per cent glycerol; in Experiment 1 the testicular tissue filtrate itself without the addition of glycerol was kept in the ice box and a similar procedure with a skin filtrate was followed in Experiment 6. The tissues were obtained from 2 spontaneous cases, rabbits Xy171 and X667, from a rabbit of the 2nd generation of the Xy171 strain, and from a rabbit of the 3rd generation of the AB18 strain. The 6 specimens of virus filtrates were injected intratesticularly in 11 rabbits in amounts ranging from 0.3 to 3.0 cc.

The results showed clinical evidence of infection in 9 rabbits; there were 5 deaths and 1 animal with outspoken symptoms was killed. The average time of death was 9.5 days after inoculation. There were 3 recoveries. In 2 rabbits, 1 each in Experiments 4 and 6, no clinical signs of infection were observed although in both cases another rabbit inoculated with the same filtrate developed pox. In Experiment 4 an animal injected with 0.4 cc. was clinically negative; the other injected with 0.8 cc. was clinically positive and was found dead on the 12th day.

Of particular interest was the result obtained with a skin filtrate derived from a spontaneous case of pox (Experiment 6). 2 rabbits inoculated intratesticularly

TABLE I

Retention of Virulence in Stored Virus Testicular Tissue Preserved in 50 Per Cent Glycerol in Ice Box. Uni- and Bilateral Intratesticular Inoculation

Experiment	Strain	Generation	Time after inoculation	Length of storage	Inoculum	Rabbits inoculated	Total dosage	Results
			days	*days*			*cc.*	*days*
1	AB18	III	7	13*	Filtered 2×	A	0.5	9 Found dead
						B	0.1	9 " "
2	Xy171	Spontaneous case	—	14	"	A	1.0	8 " "
3	"	II	4	25	"	A	2.0	Recovered
						B	1.0	"
4	"	Spontaneous case	—	28	"	A	0.8	12 Found dead
						B	0.4	No clinical evidence of infection
5	"	" "	—	32	" 2×	A	1.0	Recovered
						B	0.3	19 Found dead
6	X667	" "	—	67†	"	A	3.0	No clinical evidence of infection
						B	2.0	9 Marked orchitis. Cutaneous eruption. Killed
7	Xy171	VI	4	18	Not filtered	A	2.0	4 Found dead
						B	1.0	3 Ill. Killed
8	"	IV	8	26	" "	A	2.0	4 Found dead
						B	1.0	5 " "
9	"	XV	5	65	" "	A	1.0	4 Ill. Killed
						B	1.0	4 Found dead
10	"	III	6	72	" "	A	3.0	3 " "
						B	2.0	4 " "

* Filtrate stored in ice box without glycerol.

† Skin filtrate stored without glycerol in ice box.

TABLE I—*Concluded*

Experiment	Strain	Generation	Time after inoculation	Length of storage	Inoculum	Rabbits inoculated	Total dosage	Results
			days	*days*			*cc.*	*days*
11	Xy171	II	8	78‡	Not filtered	A	3.0	7 Moribund. Killed
						B	2.0	6 Found dead
12	"	I	7	86	" "	A	3.0	9 Critically ill. Killed
						B	2.0	6 Found dead
13	"	Spontaneous case	—	93	" "	A	3.0	6 " "
						B	2.0	5 " "
14	"	I	5	127	" "	A	2.0	4 Died
						B	1.6	4 Ill. Killed
						C	1.0	4 " "

‡ Tissue stored in taped Petri dish without glycerol.

with the fresh filtrate in doses of 0.5 and 0.2 cc. respectively developed a typical infection and died on the 7th and 14th days. The filtrate was kept in a taped flask in the ice box without the addition of glycerol or other preservative for 67 days and was then used for the inoculation of 2 rabbits in 2.0 and 3.0 cc. amounts injected (Experiment 6). In the animal injected with the larger dose no clinical evidence of infection was observed but the other developed a marked orchitis, a pronounced cutaneous eruption, and various constitutional symptoms and was killed on the 9th day. It is probable that the reason for the negative result in the former case was that the animal was an immune since it was one of the few rabbits used in the experimental work which came from the colony in which pox had occurred.

Seven specimens of unfiltered emulsions prepared from testicular tissues stored from 18 to 127 days in the ice box were injected intratesticularly in 17 rabbits. The dosage varied from 1.0 to 3.0 cc. A typical marked hemorrhagic orchitis and fever developed in each instance, and 10 of the animals died in 3 to 6 days. 5 rabbits were critically ill on the 3rd, 4th, and 9th days and were killed. The testicular tissue employed in these experiments was obtained from the original Xy171 case and from rabbits of the 1st, 3rd, 4th, 6th, and 15th generations of the serial passage of this strain. In Experiment 11 the intact testicle obtained from a 2nd generation rabbit 8 days after

inoculation was kept in a taped Petri dish without glycerol for 78 days at ice box temperature. 2 rabbits were injected with the unfiltered emulsion prepared from this tissue; one animal was found dead 6 days later and the other was moribund on the 7th day and killed.

The clinical findings of these experiments were indistinguishable from those in which fresh tissue-virus was employed, and it thus appeared that storage of tissues in glycerol at refrigerator temperature for as long as 127 days was not associated with a lowered activity of the virus. In 2 specimens stored without glycerol for 67 and 78 days, one a skin filtrate and the other testicular tissue, a potent virus was demonstrated. The findings indicate, as was also found with fresh tissue-virus inocula, that the potency of Berkefeld V filtrates was not as great as was that of unfiltered emulsions. The results on the rabbits injected with filtrates prepared from stored tissues were more irregular than were those of the unfiltered group and hence it is possible that conditions of storage lead to diminution of virus, either quantitatively or qualitatively, which is not apparent in stored unfiltered tissue emulsions.

SUMMARY AND CONCLUSIONS

A filterable agent was isolated from 7 cases of spontaneous rabbit pox by the intratesticular injection in rabbits of a variety of tissues. The virus was transmitted for 15 consecutive testicle to testicle passages in rabbits by Berkefeld V filtrates of testicular tissue emulsions. Unfiltered emulsions were more potent than filtrates. The virulence of the virus was maintained by ice box storage of infected tissues for as long as 127 days. The pathogenic properties of the virus persisted under conditions of animal passage for the 9 months from January to October, 1933, covered by these studies.

The reaction produced in rabbits by the inoculation of tissue-virus emulsions was of two principal types, the first of which has been described and analyzed in the present paper. In the case of intratesticular injections, particularly of testicular tissue inocula, an acute fulminating and rapidly fatal condition regularly developed. The outstanding features were a massive hemorrhagic orchitis with marked scrotal edema, fever, and death within a week. The second type of reaction which is taken up in the next paper of this series (1) was

observed under conditions in which the animal survived a week or longer. Occasional examples occurred in rabbits of the so called intratesticular series. The reaction was characterized by the development of a disease syndrome with a diversity of clinical manifestations which, it may be stated here for the sake of continuity, was indistinguishable from spontaneous rabbit pox.

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EXPLANATION OF PLATE 20

FIG. 1. Marked orchitis and beginning scrotal edema 3 days after bilateral testicular inoculation of 0.2 cc. Berkefeld V filtrate of testicular tissue emulsion; 3rd generation of filtrate series of Xy171 strain of pox virus. Rabbit found dead on the 6th day.

FIG. 2. Marked hemorrhagic orchitis and scrotal edema 4 days after bilateral testicular inoculation of 0.5 cc. Berkefeld V filtrate of testicular tissue emulsion; 2nd generation of filtrate series of Xy171 strain of pox virus. Rabbit found dead on the 8th day.

FIG. 3. Marked orchitis, edema of scrota and prepuce; mucous rectal cast. 8 days after bilateral testicular inoculation of 0.04 cc. Berkefeld V filtrate of testicular tissue emulsion; 2nd generation of filtrate series of Xy171 strain of pox virus. Rabbit died on the 8th day.

2
3

STUDIES ON THE ETIOLOGY OF RABBIT POX

II. Clinical Characteristics of the Experimentally Induced Disease

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PLATES 21 TO 23

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In the first paper of this series the acute and rapidly fatal type of experimental rabbit pox was described (1). The condition developed in rabbits inoculated with tissues from spontaneous cases of pox and it was characteristic also of the subsequent rabbit passage of virus with an inoculum of testicular tissue-virus and the intratesticular route of injection. In marked contrast to this fulminating type of infection was the more prolonged and less fatal disease which was observed particularly in connection with a small dosage or with routes of injection other than the intratesticular. Under these conditions, a variety of clinical manifestations developed, the most conspicuous of which was a generalized maculopapular eruption of the skin. The clinical picture of the experimental disease was a faithful reproduction of that observed in spontaneous cases of pox.

The comparisons made between the experimentally induced disease and spontaneous pox included post mortem examination of every animal inoculated with pox virus, together with microscopic examination of representative tissues. Space limitations do not permit the inclusion of this material in the present report, but it should be stated that in essential respects the pathological findings of the experimental disease and those of spontaneous pox as reported by Greene (2) were the same. The discrepancies principally concerned the degree of involvement of certain organs, but it was felt that they could be accounted for on the basis of differences in the routes of infection, in the size of infecting doses, etc.

It is the purpose of the present paper to discuss first, the clinical signs and symptoms of the experimental disease and second, certain special features of the reaction arising from different routes of inoculation.

Materials and Methods

Inoculations were in most cases carried out with tissue-virus emulsions used for the serial passages of virus maintained by the intratesticular injection of Berkefeld V filtered and of unfiltered testicular tissue emulsified in Locke's solution. The preparation of emulsions is described in the preceding paper (1). All inocula were bacteriologically controlled.

The present analysis of the clinical manifestations observed is based upon the findings on 84 male rabbits which survived for a week or longer. 61 of these animals were inoculated with the Xy171 strain of virus with which most of the experimental work was done and 23 were inoculated with 5 other strains (1). Berkefeld V filtrates were used in 72 and unfiltered emulsions in 12 cases. For 68 inoculations emulsions of testicular tissue were employed and for 16 inoculations various other tissues, namely, liver, spleen, lung, lymph nodes, defibrinized blood, heparinized blood, blood clot, and skin.

The following figures give the number of animals injected by various routes: intratesticular 38; intradermal 24; intravenous 6; intramuscular 2; intraperitoneal 2; conjunctival instillation 4; intranasal instillation 8.

The dosage varied widely. In the case of intratesticular injections, small amounts of dilutions of virus emulsions were usually employed, as for example, 0.1 cc. of a 1:1,000 dilution. Dilutions up to 1:10,000,000 were injected intradermally in 0.1 or 0.2 cc. doses. Undiluted emulsions in doses of 0.1 to 0.5 cc. were inoculated by the other routes employed.

Clinical Manifestations and Course of Disease

The analysis of the clinical manifestations of the experimental disease is based upon observations on 84 rabbits whose period of survival was a week or longer. Before taking up this analysis, however, the protocols of 2 typical cases are presented in order that the clinical picture in its entirety may be appreciated.

Both rabbits were injected intravenously with 0.5 cc. of a Berkefeld V filtrate of testicular tissue-virus emulsion of the 3rd passage of the Xy171 strain (1). An area of the body was shaved and scarified but no virus was applied. The course of the disease in both animals was similar, but whereas one succumbed, the other survived.

Rabbit A.—1st, 2nd, and 3rd day, no signs or symptoms. 4th day: profuse maculopapular eruption on shaved area not limited to scarified lines. Nodular

indurated areas in both testicles. Rectal temperature 103.8°F. 5th day: cutaneous eruption distinctly papular and hemorrhagic; size and number of papules greatly increased in shaved area (Fig. 1) and many found elsewhere, including the genito-anal regions. Pronounced orchitis with moderate scrotal edema. Popliteal adenitis. Temperature 105.7°F. 6th day: cutaneous eruption umbilicated with crust formation. Fresh papules on tip of left ear, scrota, and perineal region (Fig. 2), and on the mucocutaneous border of the lower lip and the right nostril. Thin but lively. Temperature 104.7°F. 7th day: fresh papules on the right upper lip (Fig. 3) and right upper eyelid. Blepharitis with fine scales on lids; mild conjunctivitis with a watery discharge. Cutaneous lesions markedly hemorrhagic with large necrotic centers. Very thin and weak. Temperature 103.8°F. 8th day: found dead.

Rabbit B.—1st and 2nd day: no signs or symptoms. 3rd day: area of nodular induration in left testicle. Rectal temperature 104.4°F. 4th day: widespread papular eruption on shaved area not limited to scarified lines. Temperature 105.3°F. 5th day: increase in size and number of papules on shaved areas and a similar profuse cutaneous eruption widely distributed over the body. Marked bilateral orchitis. Temperature 105.3°F. 6th day: cutaneous lesions becoming hemorrhagic and necrotic. Fresh papular eruption about the anus and on the prepuce. Popliteal adenitis. Extremely marked orchitis with scrotal edema. Temperature 105°F. 7th day: papules increasing in size and number; fresh lesions on lips. Slight watery nasal discharge. General condition good. Temperature 104.6°F. 9th day: Lesions regressing. Umbilication and crusting of papules. Temperature 102.3°F. 2 weeks: continued healing of lesions with crusted cutaneous pustules. Temperature 103.4°F. 4 weeks: negative.

With the general information derived from these protocols as a background, the clinical manifestations observed in 84 rabbits which survived a week or longer will now be discussed (Table I).

Incubation Period.—The attempt was not made to determine the exact incubation period of the disease because of the large number of rabbits required. In the case of intratesticular inoculation, some degree of local reaction was usually detected a day or so before the development of other signs.

Of the 38 rabbits injected intratesticularly with material from a variety of tissues, 33 or 94 per cent developed a definite orchitis in a mean time of 4.9 days. The earliest signs were detected on the 2nd and the latest on the 11th days. With other routes of inoculation other criteria of the incubation period were employed. The available information on the total group of animals shows that the mean times of the occurrence of fever and of a cutaneous eruption were 5.4 and 7.3 days respectively after inoculation (Table I).

TABLE I

Clinical Results in 84 Rabbits Surviving 8 or More Days. Various Inocula, Dosage, and Routes of Injection

Strain	No. of rabbits	Inoculum	Route of inoculation	Fever		Cutaneous eruption		Nasal involvement		Eye involvement		Popliteal adenitis		Orchitis		Diarrhea		Dead		Killed		Recovered
				No.	*days*	*No.*	*days*	*No.*	*days*	*No.*	*days*	*No.*	*days*	*No.*	*days*	*No.*	*days*	*No.*	*days*	*No.*	*days*	
Xy171	8	7 test. filt. 1 " not filt.	I. T.	8	4.6	6	7.8	4	6.3	5	8.2	4	5.5			1	14	6	12.0	1	9.0	1
	7	Various filt.*	"	7	7.1	4	8.0	4	9.5	1	11	4	7.3			2	6	3	14.0	—	—	4
	17	Test. "	I. D.	17	5.8	14	6.9	9	7.2	9	8.7	10	8.1	13	7.4	3	6.3	5	16.2	—	—	12
	7	" not filt.	"	7	3.0	7	5.3	6	9.5	6	9.5	1	6	3	10.7	—	—	5	9.4	2	14.0	—
	6	" filt.	I. V.	6	4.5	6	5.7	2	7.5	1	6	5	6.4	5	4.8	1	12	2	10.5	1	8.0	3
	2	" "	I. M.	2	4.0	2	6.5	1	4	1	10	2	4.5	2	6.0	—	—	—	—	—	—	2
	2	" "	I. P.	2	3.5	2	5.5	1	10	1	11	2	4.5	2	5.5	1	17	1	10.0	—	—	1
	4	" "	Conj.	4	5.3	4	6.8	—	—	—	—	3	7.3	2	7.5	—	—	—	—	—	—	4
	5	" "	Nose	5	5.6	3	7.7	—	—	—	—	3	5.3	2	10	—	—	1	10.0	—	—	4
	3	" not filt.	"	3	3	3	7	—	—	—	—	3	6.3	3	6.3	—	—	—	—	3	8.3	—
AB18	11	" " "	I. T.	11	5.8	8	8.4	4	7.0	6	7.3	6	6.8			1	11	6	9.3	1	8.0	4
P 2	4	Various filt.†	"	4	6.8	3	12	4	8.5	3	11.7	2	8.0			3	14.7	4	18.8	—	—	—
HA46	2	" " ‡	"	2	7.0	1	7	1	9	—	—	1	7			—	—	—	—	2	11.0	—
8415	2	1 test. " 1 " not filt.	"	2	6.5	—	—	1	10	—	—	2	7.5			2	7.5	1	9.0	—	—	1
X667	4	Various filt.§	"	4	7.0	4	8.5	1	10	1	10	2	10			1	12	1	14.0	1	9.0	2
Total..	84	70 filt. 14 not filt.		84	5.4	67	7.3	38	8.0	34	8.9	50	6.9	32	7.2	15	10.4	35	12.5	11	9.9	38
Incidence, *per cent*.....				100.0		79.8		52.8		47.2		59.5		69.6		17.8		41.7		13.1		45.2

I. T. = intratesticular; I. D. = intradermal; I. V. = intravenous; I. M. = intramuscular; I. P. = intraperitoneal; Conj. = conjunctival instillation.

* 2 lung, 3 heparinized blood, 1 defibrinated blood, 1 liver.

† 1 spleen, 1 lung, 1 heparinized blood, 1 blood clot.

‡ 1 testicle, 1 popliteal lymph node.

§ 2 liver, 2 skin.

Mortality.—The actual mortality rate of the group was 42 per cent and the mean time of death was 12.5 days after inoculation (Table I). Of the 35 fatalities 27, or 77 per cent, occurred on the 8th to the 14th days. The earliest deaths were on the 8th and the latest on the 28th days respectively.

Eleven rabbits were killed because of their general condition at a mean time of 9.9 days after inoculation, the earliest being on the 8th and the last on the 14th day respectively. With the inclusion of these animals, the mortality rate is raised to 55 per cent.

Recovery.—Practically half the animals of the group, that is, 38 or 45 per cent, recovered from the infection and survived (Table I).

Fever.—A rectal temperature higher than 102°F. was recorded at some period of the disease in each of the 84 rabbits in this assembled group (Table I).

The mean time of the first record of fever was 5.4 days after inoculation; the earliest instance was on the 2nd and the latest on the 9th day. Both the onset and the decline of fever were abrupt, but since readings were made only at 24 hour intervals, this feature may have been more apparent than real. The duration of fever varied considerably. In animals which developed an extensive cutaneous eruption or those with marked testicular or respiratory involvement, it was frequently present for several days. In less severe conditions, it was usually noted for 2 or 3 days.

Cutaneous Eruption.—The most conspicuous objective manifestation of the experimental disease was a maculopapular or papular eruption on the skin and mucocutaneous borders.

The eruption was observed in 80 per cent of the group at a mean time of occurrence of 7.3 days after inoculation (Table I). The earliest example noted developed 3 and the latest 14 days after inoculation. The condition began as small pinkish macules or maculopapules distributed most frequently over the back and sides of the body (Figs. 1, 5, and 6), the nape of the neck, the ears (Fig. 4), the eyelids, the muzzle, the mucocutaneous borders of the nose and lips (Figs. 3 and 11), the anus and the sheath and scrotum of male animals (Fig. 2). The number of lesions in the beginning varied from 1 or 2 to a dozen or more, but within a day or so there was frequently a countless number. The earliest lesions were very small pink or red spots which were just palpable or indurated points which were almost colorless. The size of the papules increased to a diameter of 2 or 3 mm. and in 2 or 3 days many of them became pustules with an umbilicated center and a thin yellowish crust (Figs. 4 and 6); occasionally, an intervening vesicular stage was seen. In other instances the papules became purpuric in appearance and in a

few hours were hemorrhagic and edematous with necrotic centers (Fig. 5). As a rule, individual lesions continued to be discrete but occasionally, and notably in the case of the hemorrhagic type of lesion, contiguous papules coalesced and later these areas might become secondarily infected. Regression of the eruption was usually well under way within a week of its appearance and in 2 or 3 weeks, healing was completed. In certain instances this was accompanied by scar formation; in many cases, there was no scar visible in the gross.

A striking feature of the eruption in certain animals was the development of fresh macules and papules during the evolution of the earlier lesions (Figs. 5 and 6). The new lesions appeared not only in previously uninvolved parts of the body, but also in areas already similarly affected. The number was variable, few or numerous lesions being observed. As a rule, they did not exhibit to the same degree the tendency toward umbilication and pustulation shown by the earlier lesions, and their healing consequently often occurred at the same time or might even antedate the healing of older lesions. The picture presented by a severe case at the height of the cutaneous eruption was that of macules, maculopapules, papules, and pustules, all present at one and the same time.

Nasal and Respiratory Involvement.—A nasal discharge was a prominent feature of the disease. It developed in 53 per cent of the 72 rabbits of the group inoculated by routes other than those of conjunctival or nasal instillation (Table I).

The mean time of onset was 9.5 days after inoculation. Since a number of animals were killed at this period, the true incidence rate may have been higher. The first sign was a scanty serous discharge which occurred in some animals as early as 3 days after inoculation. The condition usually increased in severity and at a fairly rapid rate so that within 3 or 4 days the discharge was profuse. By this time it was generally of a seropurulent or mucopurulent character and frequently blood stained, and about the nares there was an accumulation of reddish or brownish yellow adherent crusts (Figs. 11 and 12). The muzzle was often swollen and the skin and underlying tissue were thickened and boggy. In severe cases the respiration was labored and rapid and instances of outspoken dyspnea were not uncommon. The stance of these rabbits in which the head was held high and extended backward was very characteristic. Cases which terminated fatally had frequently shown marked respiratory involvement.

Eye Involvement.—The eyes and lids were affected in 47 per cent of 72 rabbits not inoculated by the conjunctival or nasal route (Table I).

As in the case of nasal signs and symptoms, the real incidence was probably higher since the mean time of development of clinical signs was 8.9 days after inoculation at which time many animals in the group were killed. The first evidence of eye involvement was usually photophobia and this was accompanied

or immediately followed by thickening and reddening of the lid margins, a mild conjunctivitis, a slight watery discharge, and a few tiny crusts or scales about the lids. Not infrequently the severity of the condition increased very rapidly and a keratitis with or without pannus formation, and an iritis developed. In these circumstances the discharge became seropurulent and profuse and the swollen lids became closed. Corneal ulcers and perforations were occasionally seen.

Once the cornea and iris became involved, there was little tendency toward a clearing of the condition until other manifestations of the disease were regressing. A diffuse or patchy clouding of the cornea was a frequent residual lesion long after all acute manifestations had healed.

Orchitis.—In male rabbits inoculated by routes other than the intratesticular, an orchitis was a common symptom.

Orchitis developed in 70 per cent of 46 rabbits at a mean time of 7.2 days after inoculation (Table I). The first signs of the condition were small areas of thickenings or of nodular induration which rapidly increased in size so that within 2 or 3 days the testicle was diffusely indurated or resistant and considerably enlarged (Fig. 2). In some of these cases the parenchyma became hemorrhagic and this change might be detected during life. In other cases, the initial lesions tended to remain discrete and multinodular in type. Edema of the scrotum usually accompanied the orchitis. In cases which recovered the condition regressed fairly rapidly and eventually the testicles seemed normal clinically. Not infrequently, however, small fibrous or nodular areas persisted for some time after other clinical manifestations of the disease had healed.

Lymph Adenitis.—Enlargement and increased resistance or induration of the superficial lymph nodes and particularly those of the popliteal group was a usual occurrence during the course of the experimental disease.

A popliteal adenitis was recorded in 60 per cent of 84 rabbits with a mean time of occurrence of 6.9 days after inoculation (Table I). In a considerable proportion of cases the condition was subject to fluctuations, that is, the nodes were enlarged and resistant for several days and then became much smaller and indurated only to return to their former enlarged state. From this finding, together with the post mortem results on rabbits which died or were killed at the height of the infection, it is probable than an adenitis is a constant feature of the disease at some time or other. In recovered cases, examined 3 or 4 weeks after inoculation the popliteal nodes were recorded as normal or negative.

Gastro-Intestinal Involvement.—Evidence of involvement of the gastro-intestinal tract was furnished by the character of the feces.

In the first days of the disease the stools were frequently soft and unformed and this might be the only abnormality in mild or moderately severe cases. In a considerable proportion of animals mucus was also present and formed and otherwise normal looking feces were coated with mucus. The discharge of large amounts of unstained mucus of a soft or gelatinous consistency was not uncommonly seen (Fig. 13). In some instances the mucus was firmer and more tenacious so that it had the appearance of a cast (1).[1] Very soft moist stools or an outspoken diarrhea was a fairly common occurrence in the more severe conditions and particularly in the fatal cases. In the group of 84 rabbits (Table I) a diarrhea was noted in 15 animals or 18 per cent, with a mean time of occurrence of 10.4 days after inoculation. Of the 15 rabbits with this symptom, all but one were fatal cases.

It should be mentioned for the sake of completeness that papular lesions of the mucosa of the mouth and tongue were relatively frequent. These could sometimes be detected clinically.

General Symptomology.—In addition to the clinical manifestations just described, there were others of a more general character. Obvious signs of illness were practically always observed in rabbits which developed widespread lesions.

A listless, apathetic appearance and a disinclination to move about were usual features. Not infrequently the eyelids were droopy and partly closed even in the absence of gross eye involvement. The fur became rough and unkempt. The appetite was impaired and a loss of weight was common. In the more severe cases, the rabbits rapidly became very thin, weak, and prostrated. The general appearance of many animals was one of profound intoxication. On the other hand, there were instances in which the rabbit was comparatively lively and active despite an extensive cutaneous eruption and other signs of the disease. Such cases usually recovered if respiratory involvement was not present or was not severe.

Blood Cytology.—Observations on the blood cytology showed certain well marked variations from normal values (3). After the examination of a number of rabbits at various stages of the disease had shown that the blood picture was abnormal, two groups of rabbits were studied systematically with daily counts.

In the first group, 5 rabbits were inoculated in each testicle and intradermally at two sites; all were dead on the 3rd day. In the second group, 7 rabbits inoculated in each testicle were all dead by the 5th day. The results on the two groups were sufficiently similar to be considered together.

The mean red cell count on the 1st day after inoculation was depressed from a

[1] Pearce, Rosahn, and Hu (1), Fig. 3.

preinoculation level of 5,320,000 to 4,891,000 cells, but rapidly rose to 5,230,000 on the 2nd day and on the 3rd day to 5,593,000. The mean value for the last count before death was 5,683,000. It is possible that this rise was relative and due to dehydration although the presence of numerous nucleated red cells in the peripheral blood gave evidence of bone marrow activity. The changes in the hemoglobin level paralleled those of the red cell count. A profound depression in the platelet count was observed from a mean preinoculation level of 619,000 to 495,000 on the 3rd day after inoculation. In animals that survived longer than 3 days after inoculation, the platelet count rapidly rose again to a mean value of 837,000 for 4 animals on the 4th day and a value of 1,040,000 for the single animal that survived 5 days. The total white blood cell count increased sharply on the 3rd day after inoculation from a preinoculation mean level of 6,971 to 11,765. In animals which survived the 3 day period after inoculation, the total white count rose to 15,000 and 20,000 on the 4th and 5th days. Neutrophils increased from a preinoculation mean level of 3,610 to mean values of 4,839, 6,132, 9,269 on the 1st, 2nd, and 3rd days respectively. In relative per cent this represented an increase from a preinoculation value of 52.1 per cent to 77.0 per cent. Basophils and eosinophils decreased in number, both relatively and absolutely.

The changes in the lymphocyte and monocyte values were most striking. Lymphocytes gradually fell from a preinoculation mean level of 2,473 cells comprising 35.1 per cent of the total count to 1,501 (21.2 per cent), 1,023 (13.7 per cent), and 805 (8.1 per cent) on the 1st, 2nd, and 3rd days after inoculation. Monocytes, however, after a slight decrease on the 1st and 2nd days from a preinoculation level of 374 (5.4 per cent) rose precipitously on the 3rd day to 1,382 (12.2 per cent). The mean monocyte count for 4 animals on the 4th day after inoculation was 2,026.

The changes observed in these two groups of rabbits systematically studied were duplicated in isolated examinations on other animals. It was found that the severity of the disease could be gauged with remarkable accuracy by the blood cytology findings. An increase in neutrophils, platelets, and monocytes, together with a fall in lymphocytes, were characteristic features of severe infections and in general were of bad prognostic import. At the height of the infection lymphocyte values lower than 1,000, platelet values higher than 1,000,000, and monocyte counts above 3,000 were not uncommon. On the other hand, rabbits which were recovering from the disease were observed to have decreasing values for the total white count, platelets, and monocytes, and increasing numbers of lymphocytes. Animals that recovered from severe infections usually presented a mild degree of secondary anemia, followed by a gradual return to normal values.

Miscellaneous Routes of Inoculation

The foregoing discussion on the clinical manifestations and course of disease in experimental pox was based upon an assembled group of 84 rabbits which survived for a week or more. It was pointed out that this longer survival period was associated with a small dosage and with routes of inoculation other than the intratesticular. The local and general results of particular routes of injection have hitherto not been referred to except in the case of the intratesticular route which was discussed in the preceding paper (1). Certain of these findings which present particular points of interest will now be taken up.

Both Berkefeld V filtered and unfiltered testicular tissue-virus emulsions in variable dosage were employed, the material being obtained from serial passage rabbits inoculated intratesticularly and in most instances with the Xy171 strain (1).

Intradermal Inoculation.—The intradermal injection of pox virus even in high dilutions regularly resulted in the production of marked local inflammatory lesions of the skin in which congestion, hemorrhage, edema, and necrosis were conspicuous and characteristic features. Generalized manifestations frequently developed and fever at some time or other was usually recorded. In most cases 4 or 6 virus dilutions were used, ranging from 1:10 to 1:1,000,000; amounts of 0.1 or 0.2 cc. were injected.

The character and course of the local lesion including the incubation period, rate of development, and ultimate size were directly related to dosage. The photographs of Figs. 7 to 10 inclusive illustrate typical cutaneous reactions to 4 dilutions of a *filtered* testicular tissue-virus emulsion 3, 5, 8, and 13 days respectively after intradermal injection. Doses of 0.2 cc. of the dilutions 1:10, 1:100, 1:1,000, and 1:10,000 were employed. On the 3rd day a positive reaction had developed at the site of the two largest doses (Fig. 7). On the 5th day (Fig. 8) the cutaneous lesions produced by the three largest doses were pronounced, hemorrhage and edema were present, and necrosis was beginning; in the case of the smallest dose, the lesion comprised a small slightly congested swelling. On the 8th day (Fig. 9) the lesions were very much larger, hemorrhage and necrosis had markedly increased, the edema had extended to dependent portions of the skin and subcutaneous tissues far beyond the limits of the hemorrhagic necrotic areas, and the surfaces of the three largest lesions were covered by a firm tenacious blackish red crust. The two largest lesions had coalesced. By the 10th day this area was very large and included the lesion produced by the third virus dilution of 1:1,000. At certain points the crust was beginning to break down with oozing of

a thin blood stained fluid. Along the upper margin of the area, the acute manifestations of inflammation were beginning to subside as was also the case with the lesion produced by the highest dilution of virus (1:10,000). On the 13th day the upper margin of the large crust was becoming detached and a small amount of a thick yellowish white material was exuded (Fig. 10). The lower and by far the larger portion of the lesion, however, was still a massive edematous swelling covered by a tenacious black crust and the edematous involvement of the skin and subcutaneous tissues adjacent to the lower border was more pronounced. Fever was noted on the 3rd day (105.3°F.) and on the following 5 days. A maculopapular cutaneous rash was first seen on the 4th day and by the 8th day was very profuse (Fig. 9). During the following week the eruption was more marked, individual lesions increased in size, some became hemorrhagic and umbilicated and others pustular with crust formation (Fig. 10). A nasal discharge developed on the 4th day; blepharitis and conjunctivitis, a popliteal adenitis, and a nodular orchitis were present on the 7th day. A fortnight after inoculation the animal was seriously ill and death occurred on the 20th day.

In animals which recovered the initiation of regression of the local cutaneous lesion and the duration of the process of healing depended in large measure upon the size and character of the lesion. Lesions with extensive necrosis and secondary infection were slow to heal, but healing was usually complete within a month of inoculation.

Unfiltered virus emulsions injected intradermally produced extremely marked local reactions which developed earlier and were much more extensive than those produced by corresponding doses of filtered inocula. The general character and course of the disease was likewise more severe.

The incidence and character of the principal clinical manifestations observed in 24 intradermally inoculated rabbits which survived a week or longer is given in Table I. Fever was recorded in all cases, a generalized cutaneous eruption in 21 (88 per cent), nasal and eye involvement in 15 (63 per cent), an orchitis in 16 (67 per cent), and a popliteal adenitis in 11 animals (46 per cent) respectively. 10 of these rabbits died and 2 seriously ill ones were killed, giving a mortality rate of 50 per cent. In the circumstances of the very variable dosage employed, it is not possible to say whether this value truly represents the incidence of death from intradermal inoculation. But in any event there was a sufficient number of rabbits inoculated intradermally and intratesticularly with a comparable dosage to enable one to say that the effects of intradermal injection were less severe as far as the mortality rate and the survival period were concerned.

The limits of potency of testicular tissue-virus based upon the local cutaneous reaction were not determined in a sufficient number of experiments to enable one to make more than approximate valuations of potency. With Berkefeld V filtrates in 0.2 cc. doses, lesions were produced by dilutions as high as 1:10,000 and in some instances 1:100,000. In the case of unfiltered virus, positive reactions were observed with dilutions of 1:1,000,000 and in a few instances with dilutions of 1:10,000,000.

Cutaneous Scarification.—Scarification of shaved body skin areas was carried out in many rabbits inoculated by various routes and in some cases virus was also rubbed into the scarified areas. The striking result of these procedures in which Berkefeld V filtrates were employed was the comparatively minor tendency for lesions to develop in the lines of injury despite the development of a profuse cutaneous eruption.

Typical examples of cases in which the skin was scarified but no virus was applied to the area are shown in the photograph of a rabbit taken 5 days after intravenous inoculation (Fig. 1); of another taken 9 days after intratesticular inoculation (Fig. 5); and of a 3rd case taken 8 days after intranasal instillation (Fig. 6). In a few cases lesions did develop in traumatized areas, as for example, in a small cut incurred during shaving or in lines of scarification. But such localization appeared to be a chance occurrence for the lesions were neither confined to traumatized areas nor were they more numerous there than elsewhere, while their development did not precede or exceed that of lesions in other sites. In rabbits in which filtered virus was also applied to scarified cutaneous areas, the skin along the scarified lines was reddened and thickened within 24 to 48 hours, but this change rarely persisted for more than a day or so and no other was observed.

In a group of 20 rabbits inoculated by various routes other than the intratesticular with virus filtrates and in which cutaneous areas on one or both sides of the body were scarified, a generalized maculopapular rash developed in 14 animals or 70 per cent at a mean time of 5.9 days. 9 rabbits recovered and in 8 of them a cutaneous eruption developed at a mean time of 6.6 days; 11 rabbits died at a mean time of 7.3 days and in 6 of them a papular rash developed at a mean time of 5 days. In a group of 12 rabbits inoculated intratesticularly with various amounts of filtrates and in which virus was also applied to scarified skin areas, the average time of death of 11 animals was 5.7 days; there were 4 cases of a generalized cutaneous eruption developing at a mean time of 4.8 days. In the single recovered animal, a rash was seen on the 7th day. In none of these 18 rabbits with a generalized cutaneous eruption did there appear to be any predilection for lesions to develop in the lines of scarification. From the above figures it appears that in the fatal cases as well as in those which recovered there was sufficient time for cutaneous lesions to develop in lines of scarification had there been any outspoken tendency for them to do so.

The use of *unfiltered* tissue-virus emulsion in experiments of this kind was comparatively limited. The results on animals inoculated by various routes and in which the skin was scarified but no local application of virus was made, were similar to those just described in which filtrates had been used. In the cases in which unfiltered emulsions were rubbed into scarified cutaneous areas, there was a very pronounced local reaction along the lines of scarification with swelling, edema, congestion, hemorrhage, and necrosis. The lesions developed rapidly and within a

week adjacent skin areas might be included with the formation of boggy necrotic masses resembling the lesions produced by intradermal inoculation (Fig. 14). In certain instances, some discrete lesions did develop in the lines of scarification, particularly at their ends, but on the whole this result was overshadowed or masked by the other more general lesions.

Considering the high incidence of a generalized cutaneous eruption in cases of the experimentally induced disease, the fact that similar lesions failed to develop to any extent in scarified skin areas seems curiously inconsistent. Moreover, as will presently be described, a similar failure occurred in connection with scarification of the cornea. Is it possible that the slight injury incurred by these structures from superficial scarification led to the production *in situ* or to the localization of immune principles which were sufficient to prevent the development of visible discrete lesions? Whatever the explanation may be, the effect was not only rapidly attained but was also prolonged. In so far as clinical observation was concerned, the result obtained was contrary to that observed in other conditions in which the localization of lesions at points of injury characteristically occurs.

Intranasal Instillation.—Infection was regularly accomplished by dropping testicular tissue-virus emulsion in one nostril; 14 rabbits were inoculated in this manner.

For 5[2] animals Berkefeld V filtrates were used; 4 animals which received 0.1 or 0.3 cc. recovered while 1 inoculated with 0.5 cc. was found dead on the 10th day. Unfiltered emulsions in 0.5 cc. and 0.6 cc. amounts were used for 9[3] inoculations; 3 animals were dead 3 and 6 days later and 6 were killed from 3 to 9 days after inoculation. Some of the latter rabbits might have recovered. The available though scant evidence suggests that with a comparable dosage the mortality rate after intranasal inoculation would be lower than after intratesticular inoculation.

The local clinical reaction resulting from intranasal inoculation was similar to that observed in instances of nasal involvement occurring in the course of the disease produced by other routes of injection (Figs. 11 and 12). In a typical case inoculated with 0.1 cc. of filtered virus in the right nostril, there was, 10 days later, a profuse bilateral serosanguinous discharge, more marked on the right side, a few thin crusts had formed about the nares, and the tissues of the lips and lower nose were swollen and indurated. The nasal discharge was first noted on the 4th day, fever on the 6th, eye involvement, a few cutaneous papules, and popliteal adenitis

[2] The results on these rabbits are summarized in Table I.

[3] The results on 3 of these 9 rabbits are summarized in Table I.

on the 7th, and a marked nodular orchitis on the 12th day respectively. Regression of all lesions was well under way a fortnight after inoculation and a week later the animal was practically negative.

In 13 of the 14 rabbits inoculated intranasally, fever was recorded at some time or other; it was first noted on the 5th or 6th day in the case of filtrates and on the 2nd or 3rd day in the case of unfiltered inocula. The one exception was an animal with a subnormal temperature on the 2nd day which was found dead on the 3rd day. A generalized cutaneous rash was observed in 3 of the 5 rabbits inoculated with filtrates and in 4 of the 9 inoculated with unfiltered material; the mean time of development was 7.6 days after inoculation. Omitting the 4 animals of the latter group which died or were killed 3 to 6 days after inoculation, the incidence rate of a cutaneous eruption was 70 per cent. An example of the rash in these rabbits is shown in Fig. 6; the photograph was taken 8 days after the nasal instillation of 0.3 cc. of filtered virus. Other clinical manifestations of the disease were also observed (Table I), but little can be said about their incidence or general character because of the limited number of animals.

Conjunctival Instillation.—This route of inoculation was employed in 4 rabbits; 0.1 or 0.15 cc. filtered virus emulsions were used for 5 eyes, 0.5 cc. for 1 and 0.01 cc. of a 1:10 virus dilution for another; 1 eye was not inoculated.

A well marked local reaction developed in 4 and a minor response in 2 eyes; none was observed in connection with the diluted virus. All 4 rabbits developed generalized disease manifestations, including fever which was first noted on the 3rd to the 9th day, and a maculopapular cutaneous eruption at a mean time of 6.8 days (Table I). There were no fatalities.

The first indication of a local reaction was seen on the 3rd and 4th days and comprised swelling and reddening of the lids, lachrymation, and photophobia. The condition rapidly became intensified and within a few days the lids were tightly glued together by a thick purulent secretion and about the eye was an accumulation of yellowish tenacious crusts. Separation of the lids revealed a marked conjunctivitis and diffuse keratitis. Regression of the lesion was usually well under way by the end of the 2nd week.

An acute edema of the lid conjunctiva was also seen, and in some cases it developed in the absence of other marked eye lesions. In the case of an animal in which 0.15 cc. of virus filtrate had been dropped in the conjunctival sac, edema of the lower lid conjunctiva and of the 3rd eyelid was well marked on the 4th day, but there was only slight congestion of the conjunctival vessels and slight lachrymation. On the 7th day (Fig. 16) the lesion included marked edema of the upper lid conjunctiva, marked swelling and reddening of both lids, and a mucopurulent secretion.

Various degrees of corneal involvement were usually noted after conjunctival instillation of virus, and the keratitis sometimes included pannus formation.

Corneal ulceration and perforation might subsequently occur. Examples of these lesions are illustrated by the photographs (Figs. 17 and 18) of a rabbit taken 11 and 23 days after the conjunctival instillation of 0.3 cc. of a 1:10 dilution of a virus filtrate.

All of the eye lesions which developed after conjunctival inoculation were also observed in certain rabbits inoculated by other routes. Their general character and course was the same. Healing was accomplished without residual effects except in certain cases of persistent small areas of corneal opacity or of the depressed scar of a previous ulcer. There were also some instances of scarring and distortion of the eyelids.

Scarification of Cornea and Conjunctival Instillation of Virus

Attempts to produce lesions of the cornea in lines of scarification were unsuccessful.

The cornea was anesthetized by the instillation of a 2 per cent solution of cocaine and then scarified with the point of a corneal knife. Of 4 rabbits so prepared and inoculated with virus filtrates intravenously, intramuscularly, or intraperitoneally, no gross lesions of the 8 eyes were detected; all 4 animals developed fever and a generalized maculopapular eruption, and 1 was found dead on the 10th day. In 2 other rabbits 2 drops of full strength virus filtrates were dropped on the scarified cornea of both eyes; a minor conjunctivitis developed but no corneal lesions were observed. The same procedure but with the use of unfiltered virus was carried out on 4 rabbits. A blepharitis and conjunctivitis developed on the 2nd and 3rd days, and there was a faint diffuse clouding of 2 corneas but no focal lesions confined to the lines of scarification could be made out.

Inclusion Bodies

The question of inclusion bodies in rabbit pox was first investigated in cases of the spontaneous disease (2). Microscopic examination of tissues in different stages of the infection failed to reveal any cytoplasmic or nuclear changes which were sufficiently characteristic to be called inclusion bodies. A similar negative result was obtained on tissues from experimental cases until inoculated corneas were studied. These specimens were obtained 1 to 5 days after conjunctival instillation of unfiltered virus in eyes in which corneal scarification had been carried out just prior to inoculation. The corneas themselves showed no gross change other than a faint diffuse clouding. It was found on

microscopic examination, however, that many epithelial cells contained definite cytoplasmic bodies which appeared to be identical with Guarnieri bodies (Fig. 15); they were most frequent in the deeper cells. The inclusions were found in greatest numbers in the specimens examined 48 hours after inoculation and were distinctly less numerous in older lesions.

Intravenous Inoculation.—The results of intravenous inoculation were particularly successful in the production of a pronounced clinical syndrome including an extensive maculopapular cutaneous eruption.

Berkefeld V tissue-virus filtrates were used for the 6 rabbits inoculated by this route (Table I). No intravenous injections were carried out with unfiltered emulsions. 3 rabbits which received doses of 0.2, 0.4, and 0.5 cc. respectively recovered, 2 injected with 0.4 and 0.5 cc. were found dead on the 13th and 8th days, and 1 injected with 0.4 cc. was killed on the 8th day. Each rabbit developed fever, the mean time of the first observation being 4.5 days, an extensive maculopapular cutaneous eruption (Figs. 1 and 4) at a mean time of 5.7 days, and a nodular orchitis which was detected at a mean time of 4.8 days. In 5 of the 6 rabbits a well marked popliteal adenitis was noted on an average of 6.4 days. Other less constant features were nasal involvement in 2 (Fig. 11), eye involvement in 1, and a diarrhea in 1 animal respectively.

Intramuscular Inoculation.—2 rabbits injected in the thigh muscles with 0.4 and 0.5 cc. respectively of Berkefeld V tissue-virus filtrates recovered from severe infections (Table I). There was a very marked swelling of the injected muscles. At first, the muscles felt tense, then somewhat boggy and within a few days they became very indurated. Fever was first recorded on the 3rd and 5th days and a profuse maculopapular cutaneous rash developed on the 8th and 5th days (Table I). In both animals a bilateral popliteal adenitis and orchitis with scrotal edema developed, and in one there was nasal and eye involvement including a keratitis. Regression of all lesions began in about a fortnight and healing was practically complete 3 weeks after inoculation.

Intraperitoneal Inoculation.—2 rabbits were injected intraperitoneally with 0.4 and 0.5 cc. respectively of Berkefeld V tissue-virus filtrates (Table I). Both animals developed a well marked disease from which 1 recovered while the other was found dead on the 10th day. Fever was first observed on the 3rd and 4th days, and a cutaneous rash developed on the 5th and 6th days. In both animals there was a bilateral orchitis and a popliteal adenitis. Eye involvement with keratitis, a nasal discharge, and a diarrhea were also seen in the rabbit which recovered.

SUMMARY AND CONCLUSIONS

The clinical manifestations and course of disease observed in experimental rabbit pox have been described and analyzed. The condition

differed from the acute fulminating and rapidly fatal type of experimental infection (1) in that the period of survival was longer, a variety of clinical manifestations developed and a considerable proportion of the cases recovered. The most conspicuous symptom was a generalized papular eruption on the skin and mucocutaneous borders.

The production of the disease was associated with routes of inoculation other than the intratesticular or with a small dosage. The majority of cases were inoculated with Berkefeld V filtrates of tissue-virus emulsions and not with the more potent unfiltered emulsions.

The local reactions resulting from various routes of inoculation were described. Of special interest were the pronounced cutaneous reactions induced by intradermal injection, the high instance of marked clinical manifestations after intravenous inoculation, the failure of lesions to localize in the lines of scarification of skin and cornea even in cases with a profuse cutaneous eruption, and the development of cytoplasmic inclusion bodies in the epithelial cells of the cornea following scarification and conjunctival instillation of virus.

In the character of its clinical manifestations and course of disease, experimental rabbit pox was indistinguishable from cases of the spontaneous pox.

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EXPLANATION OF PLATES

PLATE 21

FIGS. 1, 2, and 3. Intravenous inoculation of 0.5 cc. Berkefeld V filtrate of testicular tissue emulsion; 3rd generation of filtrate series of virus passage. Skin was scarified but not inoculated. Rabbit found dead on 8th day.

FIG. 1. 5 days. Profuse papular cutaneous eruption. Hemorrhage and beginning necrosis of papules.

FIG. 2. 6 days. Papules on left scrotum, prepuce, and anus. Orchitis present and scrotal edema.

FIG. 3. 7 days. Papules on right upper and lower lips and left nares.

FIG. 4. Profuse cutaneous eruption on ears. 8 days after intravenous injection of 0.4 cc. Berkefeld V filtrate of testicular tissue emulsion; 4th generation of filtrate series of virus passage. Rabbit found dead on 14th day.

FIG. 5. Recent and late cutaneous papules; older lesions hemorrhagic and umbilicated. 9 days after unilateral testicular injection of 0.2 cc. Berkefeld V filtrate of skin emulsion from spontaneous case of pox (rabbit X667-1 (1)). Rabbit found dead on 14th day.

FIG. 6. Recent and late cutaneous papules; 1 large umbilicated lesion. 8 days after intranasal instillation of 0.3 cc. Berkefeld V filtrate of testicular tissue emulsion; second generation of filtrate series of virus passage. Rabbit recovered.

PLATE 22

FIGS. 7 to 10. Cutaneous reaction 3, 5, 8, and 13 days after intradermal injection of Berkefeld V filtrate of testicular tissue emulsion; 5th generation of filtrate passage series of virus. 0.2 cc. doses of 1:10, 1:100, 1:1,000, and 1:10,000 dilutions. Rabbit found dead on 20th day.

PLATE 23

FIG. 11. Papular eruption on lips, nares, and muzzle. Marked nasal discharge; swollen muzzle. Same rabbit as Fig. 4, 11 days after inoculation.

FIG. 12. Blood stained crusts about nares. Nasal discharge. 13 days after bilateral testicular injection of 0.5 cc. Berkefeld V filtrate of defibrinated blood from spontaneous case (Xy171 (1)). Rabbit found dead on 18th day.

FIG. 13. Mucous rectal discharge. 5 days after bilateral testicular injection of 0.4 cc. Berkefeld V filtrate of testicular tissue emulsion; 4th generation of filtrate passage series of virus. Rabbit found dead on 7th day.

FIG. 14. Cutaneous reaction 8 days after inoculation of scarified skin with unfiltered testicular tissue-virus from 17th generation of virus passage. Rabbit recovered.

FIG. 15. Cytoplasmic inclusion bodies in epithelial cells of cornea. Giemsa stain. 48 hours after corneal scarification and conjunctival instillation of unfiltered testicular tissue-virus; 14th consecutive rabbit passage. × 1,000.

FIG. 16. Marked blepharitis, edema of upper lid conjunctiva, and purulent discharge. 7 days after conjunctival instillation of 0.15 cc. Berkefeld V filtrate of testicular tissue emulsion; 3rd passage generation of virus. Rabbit recovered.

FIGS. 17 and 18. Marked blepharitis, corneal clouding with pannus, and eventually corneal ulcerations. 11 and 23 days after conjunctival instillation of 0.3 cc. of Berkefeld V virus filtrate diluted 1:10; 3rd generation of filtrate series of virus passage. The right conjunctiva was also inoculated with a larger dose. Rabbit recovered.

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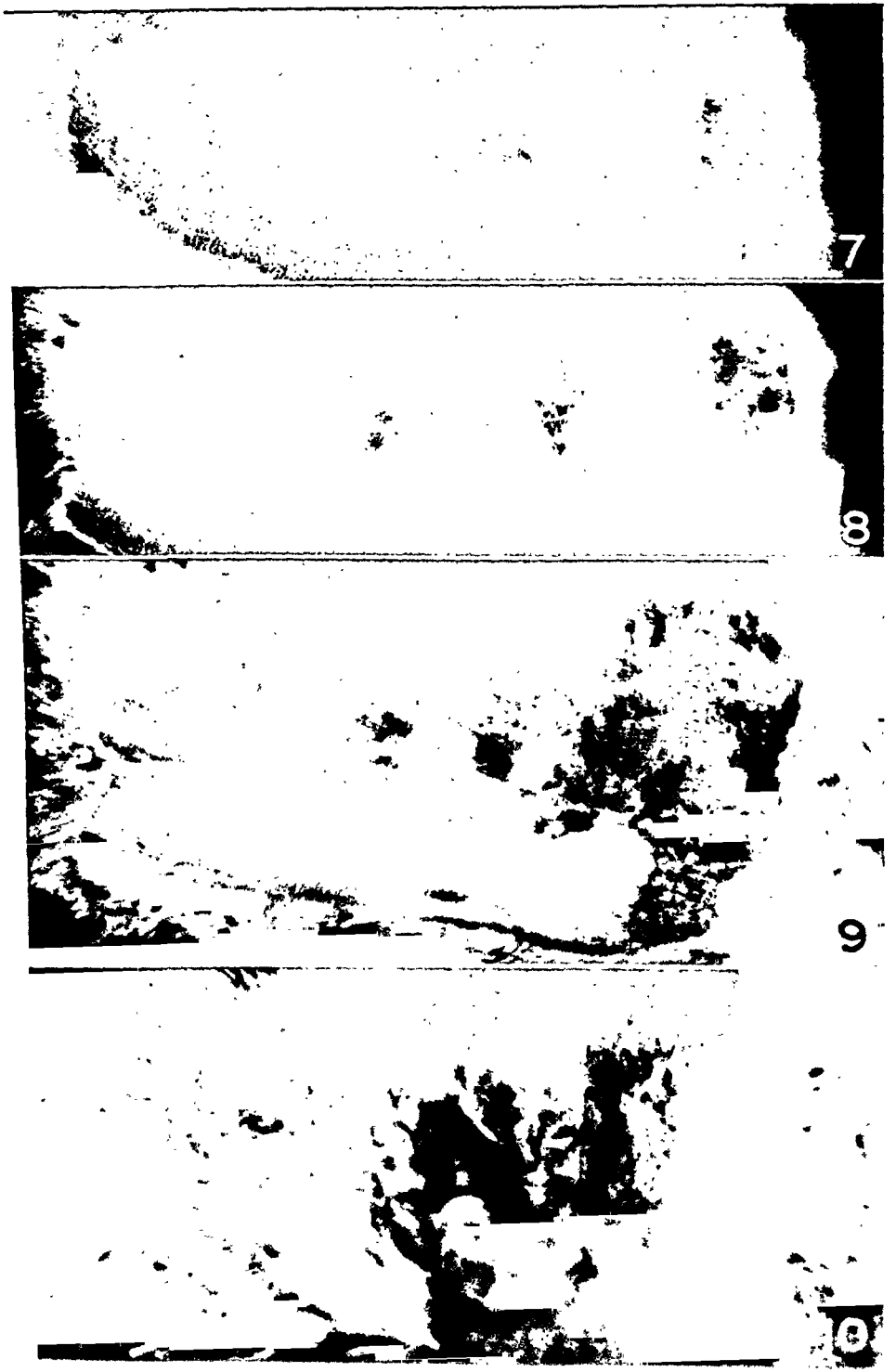
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BLOOD PLASMA PROTEIN REGENERATION CONTROLLED BY DIET*

Effects of Plant Proteins Compared with Animal Proteins The Influence of Fasting and Infection

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Our objective in this research program is a better understanding of the blood plasma proteins—particularly the materials and methods used by the body in their production to meet the emergency of plasma depletion. Just as hemoglobin production in anemia can be controlled by diet intake, in like manner plasma protein production in experimental hypoproteinemia can be controlled by diet. Moreover it is possible to regulate the production of albumin and globulin within certain limits, the plant proteins as a rule favoring globulin production. Potencies of food factors for new hemoglobin production or new plasma protein building vary widely and there is no parallelism—for example some grain proteins are very potent to regenerate plasma proteins but inert in new hemoglobin production. Chronic or acute infection can profoundly influence the production in the body of both plasma proteins and new hemoglobin. The interrelation of plasma protein formation and hemoglobin production is being kept in mind. Plasma protein can be utilized in an emergency (fasting) to maintain the body in nitrogen equilibrium (14) and evidence is accumulating to give us a better understanding of the dynamic equilibrium which obtains—an ebb and flow between plasma, organ, and tissue proteins.

The general plan of these experiments is extremely simple. The plasma proteins are depleted by removal of whole blood and the return

* We are indebted to the Eli Lilly and Co., and the California Food Concentrates Inc. for valuable material used in these experiments.

of normal red cells suspended in Locke's solution. This procedure (plasmapheresis) repeated daily will reduce the plasma protein level from 5–7 per cent (normal) to about 4 per cent, at which level there is a strong stimulus for the body to regenerate new plasma protein. This low basal level of 4 per cent plasma protein concentration is maintained as constantly as possible by means of suitable plasmapheresis and dietary control. The plasma protein removed is analyzed and credited to that particular diet period. Many complications develop in such experiments but experience is bringing them under control.

The first two papers of this series (5, 14) give information which cannot be reviewed here but we may mention one point. The normal dog has a *reserve store* of plasma protein building material which may amount to 30 to 120 gm. new protein with average values of 50 to 70 gm. This reserve store varies with the diet of the weeks preceding the plasma depletion and it is probable that a part of this reserve is to be found in the liver.

We have given much attention to the *basal ration* which was not entirely satisfactory in the earlier experiments and found that the kidney basal ration used in Tables 1 and 2 was more satisfactory than the potato-bran basal. At the same time a considerable variety of *plant proteins* was studied (Tables 1 and 3) and data were procured which show that these proteins are well utilized in building new plasma proteins.

The chance observation in Dog 34-53 is of interest. This dog while the plasma proteins were being depleted developed a severe anemia (Bartonella). After treatment and elimination of the Bartonella infestation, a favorable diet for hemoglobin regeneration was given but the plasma proteins were regenerated *before* the expected hemoglobin regeneration followed. This reaction is being studied in considerable detail.

LITERATURE

In previous communications (5, 14) some earlier papers pertaining to plasma protein regeneration have been listed. Some of the more recent papers may be mentioned. Bloomfield (2, 3) has shown that rats on low protein synthetic diets and a diet of dry carrots lose weight but do not show the progressive hypoproteinemia, ascites, and so forth, that is shown by rats on a diet of fresh carrots. He ascribes the weight loss to the inadequacy of the diets and suggests that the water content of the carrots might be the determining factor in the hypoproteinemia. Cutting and Cutter (4) have shown in experiments with normal humans on protein-free, low calorie diets that there is a slight rise in the serum protein level but a fall in the plasma volumes, so that the total circulating proteins are diminished. This reaction they believe is due principally to the low fluid intake.

Myers and Keefer (13) have shown low plasma protein levels in cirrhosis with an inverted albumin-globulin ratio and believe that the accompanying ascites depends upon the osmotic pressure of the blood plasma as well as on portal obstruction. They conjecture that the hypoproteinemia may arise from a defective formation of plasma protein and a loss of protein into the ascitic fluid. The defect may be due to alteration in the function of the liver.

Kirk (6) in producing hypoproteinemia in dogs by plasmapheresis, was unable to show an inversion of the albumin-globulin ratio. This, we believe, is due to the comparatively high animal protein diet (milk and eggs) which he used in his experiments.

Keutmann and Bassett (7) have studied plasma protein regeneration in humans suffering from Bright's disease. Their patients showed hypoproteinemia, exhaustion of reserve protein, and edema. They fed proteins known to be potent in plasma protein regeneration in dogs, and these patients showed increase in urinary protein loss, deposit of reserve protein, disappearance of edema, and little change in the plasma protein level.

Weech, Goettsch, and Reeves (16) discuss the theory of equilibrium between tissue protein and plasma protein. They describe experiments showing progressive fall of plasma proteins in dogs on a low protein diet, the fall being in the albumin fraction while the globulins remained stationary.

Lombard (8) reports the lowering of plasma proteins and reversal of the albumin-globulin ratio in acute osteomyelitis of adolescents. McKenzie and Elliott (11) report observations dealing largely with surgical infections and stress the drain on body proteins and the importance of its control by means of diet and therapy. Lustig and Mandler (9) study the content of tyrosin and cystine in the various plasma protein fractions in disease.

Methods

The methods used have been described in two previous papers (5, 14). Certain modifications have been developed which deserve mention.

Our procedure in *plasmapheresis* was to remove blood from the experimental dogs by vena puncture and immediately to inject washed red cells, obtained from healthy donor dogs, suspended in glucose-Locke's solution. The normal blood plasma protein level of 5 to 7 gm. per cent was reduced and maintained by diet and plasmapheresis at levels of 4.0 to 4.2 per cent in later experiments as contrasted with 3.5 to 3.8 per cent in the earlier ones. The higher level leaves a greater margin of safety above the edema level and presumably is still sufficiently low to act as a maximum stimulus for the production of new plasma proteins. Certainly the animals act and eat better at this slightly higher basal level. Plasmapheresis was performed almost daily. Bleedings in the earlier experiments amounted to 25 to 40 per cent of the dog's blood volume, but with the improvement in basal

diets, they were lowered to a maximum of about 15 per cent of the blood volume. There was never the slightest evidence of shock from these comparatively small bleedings, and it was not necessary to start the injection prior to the completion of the bleeding. Most of the blood exchanges were made through the superficial jugular veins, using the same needle held in position for both bleeding and injection of red cells. Clean but not sterile technique was used in plasmapheresis. It is not necessary to perform blood compatibility tests on the donor dogs and the recipients of their blood, provided anemia is not permitted to develop.

The red cell *hematocrit* was maintained as near normal as possible (45 to 50 per cent) so as to prevent any abnormalities related to anemia. Among these would be the demand for proteins to regenerate in hemoglobin. The injection of 4 to 8 per cent excess red cells was ordinarily sufficient to maintain a normal hematocrit in the experimental dogs.

One of the more significant changes of methods was in the handling of the blood removed from the dogs on basal diet. 1 ml. of saturated aqueous solution of sodium citrate per 100 ml. of blood was used as an anticoagulant. The total amount of blood removed was accurately measured, thoroughly mixed, and, instead of centrifuging all of it in 100 ml. tubes as formerly done, only about 14 ml. were poured into each of two accurately graduated hematocrit 15 ml. centrifuge tubes, stoppered with cork and centrifuged at 3000 R.P.M. for 35 minutes. Readings were made of the total volume and cell volume of each tube. The average cell percentage of the two tubes was used in computing the volume of cells and plasma in the total blood withdrawn. Analyses were run on the pooled plasma removed from the centrifugalized blood.

Various types of *basal rations* were used. The details of these rations are given in the clinical history of each dog. We were searching for a diet that was of fairly small bulk, was readily consumed, yielded a low plasma protein output so that the bleedings could be kept small, and maintained the animal in health. These diets were calculated to furnish about 80 calories per kilo of body weight. 1 gm. of a salt mixture (10) was added to each daily ration, and we believe all essential vitamins were supplied in adequate amounts. The basal ration was placed in the metabolism cage each afternoon several hours after plasmapheresis. In most instances it was rapidly consumed, but if a portion remained after several hours, the animal was spoon fed. In this way all of the ration was consumed each day.

The *test food substances* used as supplemental feedings and most of the protein containing foods in the basal rations were analyzed in the raw state for nitrogen by the macro-Kjeldahl method. The protein was calculated as 6.25 times the average nitrogen value obtained from the analysis of four to ten samples. Our protein figures checked closely with those given in the Atwater-Bryant tables (1). The total amount of test food substance was divided into 7 equal portions and 1 portion was mixed with the basal ration each day for the 7 day period. All *animal tissues* were cooked in a double boiler and were fed with the broth. The quantity of the supplemental feeding is naturally limited by the animal's ability to consume it, but even more important it must be regulated so as keep the to volume of the

bleedings necessary to maintain a low plasma protein level within a reasonable figure.

When potent *supplements* are added to the basal ration the plasma proteins rise so that larger and more frequent plasmaphereses are required to maintain the desired low level of plasma protein concentration. On an average this rise is noticed on the 3rd day of supplemental feeding and will continue into the 1st or 2nd week following, dependent upon the potency of the supplement, the ease with which it is fabricated into plasma protein, the quantity fed, and the size of the bleedings. During the periods of increased protein intake, material may be stored and is finally exhausted during the subsequent control after periods. This reaction is designated as the *"carry over."*

In calculating the *nitrogen balance*, the *loss* included the urinary N, fecal N, and the N contained in all of the blood plasma removed, and the *intake* was that received from the diet. *Fecal* N determinations were made on a week's output, kept in concentrated HS_2O_4, and analyzed by the macro-Kjeldahl method. In diets where the feces were not analyzed, the N output was estimated as 1 gm. per day. This figure is too high and increases the negative balance as given.

EXPERIMENTAL OBSERVATIONS

The tabulated experiments are all of similar character and the tables present continuous periods of observation divided into 7 day units. Rarely an exigency of the experiment required a shorter or longer period but in the tables the values are corrected to 7 days. The experimental data for each dog are presented in two tables. Tables 1 to 5 give the figures relating to the plasma protein depletion and regeneration while Tables 1-*a* to 5-*a* give the figures on nitrogen balance, nutrition, blood findings, and clinical condition. The clinical history of each dog with the specific basal diets and pertinent events accompanies the respective tables.

Preliminary blood studies of 12 *normal dogs* on the kennel diet prior to placing them on a basal diet show that the plasma protein levels vary from 4.94 to 7.75 gm. per cent, *averaging 5.79;* the albumin fraction from 3.18 to 4.28 gm. per cent, *averaging 3.74;* the globulin fraction from 1.46 to 3.50 gm. per cent, *averaging 2.05;* and the A/G ratios from 1.21 to 2.60, *averaging 1.82.* These dogs averaged 22.3 gm. total circulating plasma proteins consisting of 65 per cent albumin and 35 per cent globulin or 2.16 gm. per kilo body weight.

Tables 1 and 1-*a* show a successful experiment with no confusing complications—contrast Table 5. The dog was active and healthy during the entire period of 20 weeks, the blood hematocrit was normal

and there was a slight gain in weight in spite of bleeding (plasmapheresis) of almost 14 liters.

TABLE 1

Blood Plasma Depletion and Regeneration

Bran, Rice Polishings, Potato, and Soy Bean Compared with Liver and Spleen

Dog 34-152.

Period 7 days	Diet Kidney basal	Protein intake Total for 7 days	Protein removed			Protein removed above basal*	Potency ratio Protein intake to protein output	Blood plasma Average concentration	
			Albumin	Globulin	Total			Total protein	A/G ratio
		gm.	*gm.*	*gm.*	*gm.*	*gm.*		*gm. per cent*	
Initial sample	Kennel							5.16	1.60
	Basal (26 days)							5.40	1.08
	Dextrose (7 days)	0						4.76	1.29
1	Basal	64	11.9	10.8	22.7			4.56	1.13
2	Basal	64	5.0	5.5	10.5			4.09	0.90
3	Basal + soy bean	134	10.6	10.9	21.5	9.9	7.1	4.28	0.97
4	Basal	64	6.0	6.4	12.4			4.13	0.91
5	Basal + bran flakes	110	7.1	8.4	15.5	10.6	4.3	4.14	0.83
6	Basal	64	7.4	8.2	15.6			4.16	0.89
7	Basal	64	7.0	8.5	15.5			4.15	0.82
8	Basal + rice polishings	160	7.8	8.8	16.6	18.2	5.3	4.07	0.87
9	Basal	64	7.8	10.7	18.5			4.31	0.73
10	Basal	64	7.9	9.9	17.8			4.18	0.79
11	Basal	64	5.6	7.7	13.3			4.04	0.71
12	Basal + liver	164	9.6	12.0	21.6	15.7	6.4	4.36	0.79
13	Basal	64	8.4	10.3	18.7			4.20	0.82
14	Basal	64	5.2	6.2	11.4			4.06	0.84
15	Basal + spleen	190	10.3	9.6	19.9	12.3	10.2	4.26	1.06
16	Basal	64	7.9	8.1	16.0			4.17	0.96
17	Basal	64	5.8	6.6	12.4			4.11	0.86
18	Basal + potato	115	5.6	6.3	11.9	8.3	6.1	4.10	0.87
19	Basal	64	8.3	10.4	18.7			4.29	0.82
20	Basal	64	5.9	7.8	13.7			4.08	0.76
Total			151.1	173.1	324.2				

* Estimated basal output equivalent to 12 gm. plasma protein per week.

With this type of experiment one feels confidence in the *potency ratios* which are recorded. It is of some interest that the potency

ratios for rice polishings (5.3), bran flakes (4.3), and potato (6.1) are lower than the potency ratio of an animal tissue, liver (6.4). It would seem then that certain grain proteins under these conditions are

TABLE 1-*a*

Nitrogen Balance, Blood Findings, and Clinical Condition

Dog 34-152.

Period 7 days	Diet Kidney basal	Diet Supplement per period	Weight	Negative N balance	Urinary N	R.B.C. hematocrit	Plasma volume	Total blood withdrawn
		gm.	*kg.*	*gm.*	*gm.*	*per cent*	*ml.*	*ml.*
Initial	Kennel		12.2			45.9	737	45
sample	Basal (26 days)		12.1			47.3	615	48
	Dextrose (7 days)		11.0			48.4	543	33
1	Basal	0	11.2	5.8	9.6	50.0	—	889
2	Basal	0	11.5	3.9	9.9	49.5	663	453
3	Basal + soy bean	175	11.7	+2.2	13.0	49.8	550	928
4	Basal	0	12.0	5.2	10.7	47.8	630	524
5	Basal + bran flakes	350	12.4	+0.1	12.3	48.4	542	655
6	Basal	0	12.3	5.2	10.1	47.0	—	670
7	Basal	0	12.5	4.9	9.9	46.4	672	642
8	Basal + rice polishings	525	12.9	+4.5	15.7	46.0	—	695
9	Basal	0	13.1	5.8	10.3	48.9	649	713
10	Basal	0	13.1	5.3	9.9	52.0	607	795
11	Basal	0	13.1	3.8	9.1	48.4	619	589
12	Basal + liver	500	13.3	+5.7	14.4	46.2	654	847
13	Basal	0	13.3	5.6	10.0	52.4	627	797
14	Basal	0	13.2	4.9	10.5	47.7	599	504
15	Basal + spleen	700	13.4	+9.6	14.8	46.7	660	831
16	Basal	0	13.6	5.5	10.4	46.9	665	678
17	Basal	0	13.4	5.7	11.2	50.5	584	512
18	Basal + potato	690	13.8	+1.3	12.4	45.9	695	519
19	Basal	0	13.6	7.6	12.1	52.8	608	804
20	Basal	0	13.6	5.8	11.1	46.8	584	621
Total or Average.						48.5	625	13,792

utilized a little more efficiently than the liver protein—while it requires 6.4 gm. of liver protein it requires but 5.3 gm. of rice polishings protein to regenerate 1 gm. of new plasma protein in this dog.

Kidney when added to a potato-bran basal ration (14) gives a low potency ratio (20.8) but when used in a different basal diet as in this dog is very well utilized and the potency ratio for this kidney basal is

5.3. The supplement of kidney added to the kidney basal has not yet been tried.

Liver apparently gives the same potency ratio when added to the kidney basal or the potato-bran basal ration (Table 6).

Spleen is fairly well utilized and falls into the class of casein.

The preliminary kidney basal diet period of 26 days followed by a week of fasting exhausted almost all of the reserve store of plasma protein building material and the base line output was reached by the 2nd week after fasting.

Soy bean meal (Table 1) differs in many ways from other plant or grain proteins. It is well utilized and shows a potency ratio of 7.1; moreover it is utilized very promptly and the increase in plasma protein shows within 24 hours after soy bean is added to the diet. Also, there is little of the carry over into succeeding weeks that is so obvious with potato feeding or the rice polishings supplement to the basal diet.

This dog (34-152, Table 1-*a*) showed an initial circulating plasma protein mass of about 38 gm., but after the fasting week a plasma protein mass in the circulating blood of 26 gm. This is the usual reaction and indicates the utilization of about 12 gm. plasma protein which may be looked upon as a part of the reserve protein store.

The albumin-globulin ratio started at 1.6 but was reduced to 1.13 during the first week of plasmapheresis (Table 1). After this time the A/G ratio was below 1 and fell to 0.73 with the feeding of rice polishings. Obviously the grain proteins as a rule favor the production of more globulin than albumin. Soy bean meal is an exception—compare Table 3.

Clinical History.—Dog 34-152 (Tables 1 and 1-*a*). An adult male hound weighing 12.2 kg. had been on kennel diet for several months. The plasma protein level was 5.16 per cent, albumin 3.18, and globulin 1.98 (A/G ratio of 1.6). The dog was placed on a basal ration consisting of 140 gm. cane sugar; 11 gm. butter fat; 31 gm. lard; 18 gm. Squibb's Vitavose (2.7 gm. protein); 5 gm. bone ash, 2 gm. salt mixture. This furnished a total of only 2.7 gm. protein and 83 calories per kilo body weight per day. The diet was refused after a week; consequently 150 gm. (raw weight) of cooked pork kidney (24.3 gm. protein) was added to the diet, which was then consumed readily. Then followed a week of fasting during which water was always available and 60 gm. of dextrose in 150 ml. of water was

given daily by stomach tube. The dog was returned to a basal daily diet similar to that formerly fed but somewhat modified: 50 gm. (raw weight) cooked pork kidney (8.1 gm. protein); 120 gm. cane sugar; 25 gm. canned tomato (0.3 gm. protein); 15 gm. cod liver oil; 30 gm. lard; 10 gm. butter fat; 5 gm. bone ash; 5 gm. Squibb's Vitavose (0.75 gm. protein); 1 gm. salt mixture. This diet furnished a total of 9.15 gm. protein, about 90 calories per kilo body weight daily, had a bulk of only 200 ml., and was readily consumed. There was a slight gain in weight during the next 20 weeks. Food consumption was uniformly 100 per cent. The non-protein nitrogen varied from 13 to 18 mg. per cent throughout this experimental period. Fecal analysis during basal periods showed a nitrogen loss of 2.7 gm. per week. Plasmapheresis was begun at the close of the fasting week and continued for the next 20 weeks and the dog was in excellent clinical condition throughout this period. Soy bean meal used was the cheap dairy feed material cooked into a mush. Vitavose is described as a malted wheat germ extract rich in vitamin B. "Post 40% bran flakes" is a commercial product stated to be "bran and other parts of wheat with malt syrup, sugar and salt." Rice Polishings were supplied by the Lilly Research Laboratories. Irish potato powdered and dehydrated, obtained from the California Vegetable Concentrates Inc., was used. It contained in the dry state 7.4 per cent protein.

Tables 2 and 2-*a* (Dog 33-11) give the data on an uncomplicated experiment lasting 5 weeks. The dog was in perfect health throughout and food consumption was 100 per cent. The kidney basal ration as in Table 1 was satisfactory and gave the same potency ratio (5.3), showing a good utilization of the various proteins included in the diet. Almost all the protein was derived from pig kidney (8.1 gm. protein) with 1.05 gm. protein from Vitavose and tomato.

Brain shows a potency ratio of 11.8 which puts it well down in the list. A total of 1225 gm. pork brains coagulated by heat was fed during period 3—Table 2.

The A/G ratio in this dog never dropped below 1 and this reaction is in contrast to Dog 34-152—Table 1, on the identical ration. Some dogs can produce more albumin on a given ration and this has been observed in other experiments (14). Also some dogs can produce more hemoglobin during anemic periods on a fixed diet when compared even with litter mates. This dog is being studied at the present time and more information on this point will be available.

Using this kidney basal ration gives a high carbohydrate intake but it does not cause glycosuria in these dogs. Following plasmapheresis there may be a little sugar in the urine probably accounted for by the

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given daily by stomach tube. The dog was returned to a basal daily diet similar to that formerly fed but somewhat modified: 50 gm. (raw weight) cooked pork kidney (8.1 gm. protein); 120 gm. cane sugar; 25 gm. canned tomato (0.3 gm. protein); 15 gm. cod liver oil; 30 gm. lard; 10 gm. butter fat; 5 gm. bone ash; 5 gm. Squibb's Vitavose (0.75 gm. protein); 1 gm. salt mixture. This diet furnished a total of 9.15 gm. protein, about 90 calories per kilo body weight daily, had a bulk of only 200 ml., and was readily consumed. There was a slight gain in weight during the next 20 weeks. Food consumption was uniformly 100 per cent. The non-protein nitrogen varied from 13 to 18 mg. per cent throughout this experimental period. Fecal analysis during basal periods showed a nitrogen loss of 2.7 gm. per week. Plasmapheresis was begun at the close of the fasting week and continued for the next 20 weeks and the dog was in excellent clinical condition throughout this period. Soy bean meal used was the cheap dairy feed material cooked into a mush. Vitavose is described as a malted wheat germ extract rich in vitamin B. "Post 40% bran flakes" is a commercial product stated to be "bran and other parts of wheat with malt syrup, sugar and salt." Rice Polishings were supplied by the Lilly Research Laboratories. Irish potato powdered and dehydrated, obtained from the California Vegetable Concentrates Inc., was used. It contained in the dry state 7.4 per cent protein.

Tables 2 and 2-*a* (Dog 33-11) give the data on an uncomplicated experiment lasting 5 weeks. The dog was in perfect health throughout and food consumption was 100 per cent. The kidney basal ration as in Table 1 was satisfactory and gave the same potency ratio (5.3), showing a good utilization of the various proteins included in the diet. Almost all the protein was derived from pig kidney (8.1 gm. protein) with 1.05 gm. protein from Vitavose and tomato.

Brain shows a potency ratio of 11.8 which puts it well down in the list. A total of 1225 gm. pork brains coagulated by heat was fed during period 3—Table 2.

The A/G ratio in this dog never dropped below 1 and this reaction is in contrast to Dog 34-152—Table 1, on the identical ration. Some dogs can produce more albumin on a given ration and this has been observed in other experiments (14). Also some dogs can produce more hemoglobin during anemic periods on a fixed diet when compared even with litter mates. This dog is being studied at the present time and more information on this point will be available.

Using this kidney basal ration gives a high carbohydrate intake but it does not cause glycosuria in these dogs. Following plasmapheresis there may be a little sugar in the urine probably accounted for by the

Locke's solution with 5 per cent glucose given intravenously to accompany the introduced red cells.

Clinical History.—Dog 33-11 (Tables 2 and 2-*a*). An adult female bull mongrel, born in our animal house Nov. 25, 1932. It was raised on a salmon bread apricot diet for anemia studies, was kept at an anemic level from Dec. 20, 1933, to Feb. 20, 1934, was allowed to return to normal and fed the hospital table scraps kennel ration until Feb. 19, 1935, when it was transferred to the plasmapheresis colony weighing 13.1 kg. The plasma protein level was then 5.34 per cent, 3.56 per cent albumin and 1.78 per cent globulin (A/G ratio of 2.05). The dog was placed on a

TABLE 2

Blood Plasma Depletion and Regeneration

Kidney Basal Diet and Brain Supplemental Feeding

Dog 33-11.

Period 7 days	Diet Kidney basal	Protein intake Total for 7 days	Protein removed			Protein removed above basal*	Potency ratio Protein intake to protein output	Blood plasma Average concentration	
			Albumin	Globulin	Total			Total protein	A/G ratio
		gm.	*gm.*	*gm.*	*gm.*	*gm.*		*gm. per cent*	
Initial	Kennel							5.34	2.05
sample	Basal (65 days)							5.04	1.23
	Dextrose (7 days)	0						5.21	1.14
1	Basal	50	9.8	8.9	18.7			4.64	1.12
2	Basal	64	7.0	6.8	13.8			4.10	1.00
3	Basal + brain	198	12.9	9.9	22.8	11.3	11.8	4.55	1.34
4	Basal	64	8.1	5.6	13.7			4.24	1.46
5	Basal	64	6.1	4.8	10.9			4.04	1.26
Total			43.9	36.0	79.9				

* Estimated basal output equivalent to 12 gm. plasma protein per week.

potato-bran basal ration identical to that fed to Dog 33-333 (Table 4). It furnished a total of 10.3 gm. of protein per day and approximately 85 calories per kilo body weight. After 65 days of this diet the plasma protein was 5.04 per cent, albumin 2.78 per cent, globulin 2.26 per cent (A/G ratio 1.23), and the weight was 11.6 kg. Then followed a 7 day period of fasting during which water was always available and 50 gm. of dextrose in 150 ml. of water were given daily by stomach tube. The diet then given was the kidney basal ration containing 50 gm. kidney per day as fed to Dog 34-152 (Table 1). The new diet furnished a total of 9.15 gm. of protein per day, about 90 calories per kilo, and the dog made a slight gain in weight in the next 5 weeks. The dog was in excellent clinical condition

at all times. The blood plasma non-protein nitrogen was low after fasting (10 mg. per cent) and rose to 19 mg. per cent after brain feeding.

Tables 3 and 3-*a* (Dog 34-53) while brief and incomplete give experimental data of some interest. It is obvious that the *soy bean* meal ration favors very rapid plasma protein regeneration. This supports the experiment with soy bean meal in Table 1. The high A/G ratio is well shown and contrasts with the fall during period 5 with the dog on the potato-bran basal diet. It would seem that soy bean like the animal proteins favors albumin production in contrast

TABLE 2-*a*

Nitrogen Balance, Blood Findings, and Clinical Condition

Dog 33-11.

Period 7 days	Diet Kidney basal	Diet Supplement per period	Weight	Negative N balance	Urinary N	R.B.C. hematocrit	Plasma volume	Total blood withdrawn
		gm.	*kg.*	*gm.*	*gm.*	*per cent*	*ml.*	*ml.*
Initial sample	Kennel		13.1			54.8	376	50
	Basal (65 days)		11.6			52.1	454	94
	Dextrose (7 days)		11.2			50.7	—	43
1	Basal	0	11.1	7.6	9.8	48.1	508	713
2	Basal	0	11.2	3.6	8.8	51.5	—	606
3	Basal + brain	1225	11.6	+11.1	14.2	50.3	398	918
4	Basal	0	11.9	3.5	8.7	53.0	440	621
5	Basal	0	12.1	4.0	9.7	47.8	535	470
Total or Average.....						50.1	452	3515

to the grain proteins which favor the production of globulin. This dog also shows the enormous bleedings which will be tolerated under these conditions (daily bleedings of 35 per cent total blood volume). In our experience when one continues such heavy bleedings for long periods there is apt to be trouble and the Bartonella infection in this dog probably was due largely to a lowered resistance related to the heavy bleeding.

The *potency ratio* for the soy bean diet is given as 4.6+ which means that we believe the reserve store of protein building material contributed to the total protein removed—compare this with the potency ratio (7.1) for soy bean in Table 1.

TABLE 3

Blood Plasma Depletion and Regeneration

Soy Bean Meal Favorable for Regeneration

Dog 34-53.

Period 7 days	Diet	Protein intake Total for 7 days	Protein removed			Potency ratio Protein intake to protein output	Blood plasma Average concentration	
			Albumin	Globulin	Total		Total protein	A/G ratio
		gm.	*gm.*	*gm.*	*gm.*		*gm. per cent*	
Initial	Kennel						7.27	1.21
sample	Soy bean basal (2 months)						5.37	1.87
1	Soy bean basal	173	17.3	10.8	28.1		5.17	1.62
2	Soy bean basal	173	20.5	16.6	37.1		4.65	1.22
3	Soy bean basal and fasting (5 days)	43	11.1	10.1	21.2	4.6±	4.19	1.12
4	Soy bean basal	131	14.2	12.5	26.7		4.08	1.14
5	Potato-bran basal	77	9.4	9.5	18.9	4.1±	4.04	0.98
Total			72.5	59.5	132.0			

Discontinued due to Bartonella infection.

TABLE 3-*a*

Nitrogen Balance, Blood Findings, and Clinical Condition

Dog 34-53.

Period 7 days	Diet	Weight	Negative N balance	Urinary N	R.B.C. hematocrit	Plasma volume	Total blood withdrawn
		kg.	*gm.*	*gm.*	*per cent*	*ml.*	*ml.*
Initial	Kennel	11.5			49.7	451	128
sample	Soy bean basal (2 months)	10.8					197
1	Soy bean basal	11.0	3.2	19.3	41.7	497	941
2	Soy bean basal	10.7	9.0	23.6	45.1	427	1328
3	Soy bean basal and fasting (5 days)	10.0	18.6	15.0	45.5	421	828
4	Soy bean basal	10.0	11.0	20.5	48.1	447	1100
5	Potato-bran basal	10.3	12.1	14.3	41.3	469	790
Total or Average					45.2	452	5312

A *hemolytic crisis* and *Bartonella infection* prevented further plasmapheresis. During the hemolytic episode the hematocrit dropped to a low of 18.5 per cent regardless of the injection of a large excess of red

cells. Following arsphenamine sterilization of the Bartonella infection on a diet rich in liver the plasma proteins mounted towards the normal level but the hematocrit rise was slow by comparison. Evidence is accumulating to the effect that hemoglobin is slow to regenerate in the presence of depleted plasma protein. This may mean that plasma protein takes precedence over hemoglobin when both proteins are depleted and a favorable food intake favors regeneration.

Clinical History.—Dog 34-53 (Tables 3 and 3-*a*). An adult male mongrel poodle weighing 11.5 kg. Following several months of table scraps kennel diet the plasma protein level was 7.27 per cent, albumin 3.98 per cent, globulin 3.29 per cent (A/G ratio 1.21). The dog was placed on a daily basal ration consisting of 70 gm. (dry weight) soy bean meal cooked into a thick mush with water (28.0 gm. protein); 60 gm. Karo syrup; 60 gm. canned tomato (0.7 gm. protein); 30 ml. cod liver oil; and 1 gm. salt mixture. This furnished a total of 28.7 gm. of protein and about 75 calories per kilo body weight per day. The soy bean meal was then cut to 60 gm. (24.0 gm. protein) per day, the caloric intake was adjusted to 75 per kilo body weight, and *plasmapheresis was started.* Following more than 2 weeks of heavy bleeding, during which as much as 20 to 35 per cent of the total blood volume was removed daily, and 5 days of fasting with large daily blood exchanges, in an unsuccessful attempt to deplete the large reserve store of protein, the quantity of soy bean meal in the diet was decreased to 45 gm. daily (18 gm. protein). One more week of large daily blood exchanges convinced us that soy bean meal was too potent a protein builder to make a satisfactory basal ration. The dog tolerated the large bleedings with no signs of discomfort and relished the diet. In period 5, the ration was changed to a potato-bran combination identical to that given to Dog 33-333 (Table 4) which furnished 10.3 gm. of protein and 90 calories per kilo body weight. The blood non-protein nitrogen ranged from 19 to 24 mg. per cent.

The red cell hematocrit had been maintained at 45 to 50 per cent but at the end of period 5 it had dropped to 41.3 per cent in spite of replacement of red cells ordinarily adequate to maintain the normal level. Concomitant with the drop in the hematocrit, the plasma showed hemolysis and the animal vomited bloody mucus. Injections of blood appeared to increase the hemolysis so plasmapheresis was discontinued. Kidney, liver, and Lextron (primary and secondary anemia liver fractions plus iron) were added to the diet. The hematocrit fell to 18.5 per cent. At this time *Bartonella bodies* were demonstrated in blood smears (reported elsewhere (12)). Apparent sterilization was effected by a single intravenous injection of 15 mg. of neoarsphenamine per kilo body weight.

Tables 4 and 4-*a* (Dog 33-333) give information of value related to the proteins of rice, potato, and bran. This dog for 15 weeks was on a

total of 9.11 gm. of protein and about 65 calories per kilo body weight per day. Subsequently the rice was increased to 150 gm. (9.62 gm. protein), the bran was discontinued, and the caloric intake raised to 85 calories per kilo by increasing the carbohydrates and fats. After 23 days of rice diet the plasma protein level was 6.09 per cent with an A/G ratio of 0.90.

Plasmapheresis was started at this time. Toward the end of period 2 the rice diet was only partially consumed and it became necessary to substitute a potato-bran ration. This basal ration consisted of 200 gm. boiled Irish potato (5.0 gm. protein); 35 gm. Post's bran flakes (4.6 gm. protein); 60 gm. canned tomato (0.7 gm. protein); 65 gm. Karo syrup; 25 ml. cod liver oil; 25 ml. cottonseed oil; and 1 gm. salt mixture. It furnished a total of 10.3 gm. of protein and about 90 calories per kilo body weight per day. During period 5, the dog was fasted 7 days without the usual dextrose by stomach tube.

On the 2nd day of period 6 the dog appeared ill and ate poorly. 2 days later a large abscess on the right side of the neck was opened. The plasma proteins had dropped to 3.08 per cent with an A/G ratio of 0.70. A whole blood transfusion of 210 ml. and several intravenous injections of 10 per cent dextrose (total of 160 gm.) were given. After several days of rest *plasmapheresis was resumed.* Thus period 6 covers 10 days but is recorded as a 7 day period, the proper corrections being made. The basal ration was eaten the last 5 days of this period. Periods 7 and 8 were satisfactory. For periods 9 and 10 the ration was changed to a rice basal diet similar to that fed in the beginning but containing 125 gm. (dry weight) of boiled rice (8.01 gm. protein) and sufficient of the other items to furnish 100 calories per kilo body weight per day. It was consumed satisfactorily. For period 11 the basal ration was changed to one composed of 125 gm. (raw weight) of yellow corn meal cooked into a thick mush with water (11.69 gm. protein); 65 gm. Karo syrup; 60 gm. tomato (0.7 gm. protein); 25 ml. cod liver oil; 15 ml. cottonseed oil; and 1 gm. salt mixture. It furnished a total of 12.39 gm. of protein and about 105 calories per kilo body weight per day. This diet was eaten 100 per cent, but slowly and apparently without relish.

Although the animal had been getting 90 to 100 calories per kilo body weight per day for many weeks it had steadily lost weight from 13.3 to 9.7 kg. On the last day of period 11 a severe cellulitis and edema developed in the neck and the dog was killed with ether. The non-protein nitrogen varied from 9 mg. when fasting to 17 mg. per cent on various diets. At *autopsy* the neck tissues were edematous and sections showed necrosis, abscess formation, hemorrhage, and edema. The musculature was normal but there was very little body fat. The viscera were normal except for the pigment deposits in the spleen, liver, and lymph nodes as usually noted in plasmapheresis animals.

Table 5 shows many confusing factors and illustrates some unfortunate complications which may cause trouble in these experiments. Not until period 10 did it become apparent that this dog's true basal output on the potato-bran basal diet was about 10 gm. plasma protein

per week. This means a large reserve store of protein building materials was not exhausted by the first 4 weeks of plasma depletion and this reserve store surely amounted to more than 55 gm. potential protein. Consequently the figures representing the response to *beef blood* feeding are too high as they probably include some of this reserve protein and the potency ratio is too small.

Fasting (dextrose feeding) periods 8 and 9 are of great interest and show that this dog could yield 11 gm. plasma proteins while the plasma protein concentration is a constant. About 3 gm. is to be accounted for by the shrinkage of plasma volume which always is observed in this type of experiment. This means that this dog can produce from its body proteins about 4 gm. plasma protein a week. This is to be compared with a basal output of 10 gm. plasma protein or the favorable output on a stomach feeding (period 18) of 24 gm. This emphasizes again the dependence of the body on an intake of food proteins to produce significant amounts of new plasma protein.

Infection with intoxication will cause a real drop in plasma protein production—compare period 16, Table 5. A neck abscess caused clinical disturbance, some loss of appetite, but food consumption was adequate to maintain body weight. We see a plasma protein output of only 3.2 gm. per week and during this period the dog was given some animal protein besides the potato-bran basal (see clinical history Dog 33-324). We believe this is not wholly due to impaired absorption as there was no significant weight loss and no gastro-intestinal disturbance but due largely to a disturbance of internal metabolism.

The *yeast* experiment (period 14, Table 5) is of interest and we cannot give any adequate explanation. This yeast food mixture gave some fermentation with gastro-intestinal disturbances and we observe very low plasma protein outputs on the week of feeding and the subsequent week. The urinary nitrogen is practically as low as during a fasting period but there is no weight loss which indicates some food absorption. We hesitate to suggest that any food material may actually *inhibit* the production of plasma proteins and it may be wholly a matter of absorption but it calls for further study.

Beef stomach feeding is of interest in certain anemic states so it seemed that it should be tested under these conditions. The first experiment was complicated by a brief superficial infection of the groin so that the experiment was repeated in period 18, Table 5. This

fresh cooked beef stomach favors the production of albumin over globulin as do so many of the animal proteins and raises the A/G ratio

TABLE 5

Blood Plasma Depletion and Regeneration

Infection, Fasting, Stomach and Yeast Feeding

Dog 33-324.

Period 7 days	Diet Potato-bran basal	Protein intake Total for 7 days	Protein removed			Protein removed above basal*	Potency ratio Protein intake to protein output	Blood plasma Average concentration	
			Albumin	Globulin	Total			Total protein	A/G ratio
		gm.	*gm.*	*gm.*	*gm.*	*gm.*		*gm. per cent*	
Initial sample	Kennel							5.92	2.60
1	Basal	55	16.5	10.8	27.3			4.93	1.53
2	Basal	55	11.0	9.0	20.0			4.37	1.22
3	Basal	55	13.5	10.1	23.6			4.10	1.19
4	Basal	55	11.3	11.0	22.3			3.84	0.89
5	Basal + beef blood	182	16.5	14.7	31.2	40.±	3.2±	4.04	1.12
6	Basal	55	10.1	9.2	19.3			3.79	1.08
7	Basal	55	9.2	10.3	19.5			3.89	0.88
8	Dextrose	0	2.3	2.5	4.8			3.83	0.92
9	Dextrose	0	2.9	3.1	6.0			3.86	0.91
10	Basal	55	4.9	5.4	10.3			3.72	0.93
11†	Basal + stomach	283	5.9	5.5	11.4	16.8	13.6	4.69	1.01
12	Basal	55	13.0	11.8	24.8			4.17	1.09
13	Basal	55	5.4	5.2	10.6			3.47	1.05
14	Basal + yeast	114	3.1	2.9	6.0			3.51	1.02
15	Basal	55	3.1	3.1	6.2			3.40	0.99
16‡	Mixed	?	1.6	1.6	3.2			3.82	1.13
17	Basal	55	4.7	4.8	9.5			3.71	0.93
18	Basal + stomach	283	13.3	10.7	24.0	16.7	13.6	4.25	1.17
19	Basal	55	7.4	5.3	12.7			3.93	1.44
Total			155.7	137.0	292.7				

* Estimated basal output equivalent to 10 gm. plasma protein per week.

† Gas bacillus infection.

‡ Neck abscess.

above 1. in both experiments. The potency ratio is 13.6. The potato-bran *basal ration* has a better potency ratio, 5.5.

The infections and death of this dog illustrate the fact that it is dangerous to carry the plasma depletion to a level below 3.7 gm. protein per 100 cc. If infection develops this level will fall even more, due in part possibly to escape of circulating protein. This is a vicious

TABLE 5-*a*

Nitrogen Balance, Blood Findings, and Clinical Condition

Dog 33-324.

Period 7 days	Diet Potato-bran basal	Diet Supplement per period	Weight	Negative N balance	Urinary N	R.B.C. hematocrit	Total blood withdrawn
		gm.	*kg.*	*gm.*	*gm.*	*per cent*	*ml.*
Initial sample	Kennel	0	11.2			53.0	114
1	Basal	0	10.6	13.4	10.7	50.9	1157
2	Basal	0	10.4	12.2	10.7	54.6	988
3	Basal	0	10.2	13.8	10.7	53.0	1216
4	Basal	0	10.2	12.6	10.7	48.5	1043
5	Basal + beef blood	140	10.2	6.2	23.2	52.7	1582
6	Basal	0	10.1	12.0	10.6	45.6	930
7	Basal	0	10.1	9.7	8.3	55.1	1005
8	Dextrose	420	9.7	14.1	6.8	44.5	237
9	Dextrose	420	9.2	12.7	5.2	44.8	280
10	Basal	0	8.9	8.8	8.9	49.6	680
11*	Basal + stomach	1400	9.4	+21.3	15.1	44.4	388
12	Basal	0	9.4	10.5	8.2	50.8	1094
13	Basal	0	9.3	7.2	7.3	54.0	571
14	Basal + yeast	454	9.2	+3.8	6.5	43.1	290
15	Basal	0	9.3	4.6	5.4	54.9	324
16†	Mixed	?	9.2	—	—	—	231
17	Basal	0	9.0	7.1	7.3	48.0	430
18	Basal + stomach	1400	9.2	+19.8	14.5	44.8	951
19	Basal	0	9.4	9.6	9.3	46.6	550
Total or Average.						49.0	14,061

* Gas bacillus infection.

† Neck abscess.

circle because the lower values favor more transudation through the capillary membranes. These dogs after long periods of plasma depletion show a lowered resistance to infection and this must be kept in mind in handling these animals.

Clinical History.—Dog 33-324. An adult male mongrel terrier weighing 11.2 kg. had been on kennel diet for many months. The plasma protein level was 5.92 per cent, albumin 4.28 per cent, and globulin 1.64 per cent (A/G ratio of 2.60). The dog was placed on a daily basal ration consisting of 100 gm. boiled Irish potato (2.5 gm. protein); 35 gm. Post's 40% bran flakes (4.6 gm. protein); 50 gm. Karo syrup; 60 gm. canned tomato (0.7 gm. protein); 25 ml. cod liver oil; and 1 gm. salt mixture. This furnished a total of 7.8 gm. of protein and about 55 calories per kilo body weight per day. After 2 weeks the plasma protein level was 5.53 gm. per cent with an A/G ratio of 1.93.

Plasmapheresis was started at this time. *Beef blood* (Table 5, period 5) represents a feeding of "proteins precipitated from defibrinated beef blood at 85°C in which 1 gm. is equivalent to about 4.5 ml. of blood, No. E 599, The Lilly Research Laboratories." It is a finely powdered material resembling cocoa which is 90.7 per cent protein by our analyses and was readily eaten when mixed with the basal ration in the raw state. The potency ratio of 3.2 is too high since the true basal output level had not yet been reached and a portion of the output credited to the beef blood belongs to the reserve store. Beef serum added to this same basal diet showed a potency ratio of 2.6 (14). During fasting periods 8 and 9, Table 5, the dog received 60 gm. dextrose each day in about 150 cc. water. At the beginning of the 11th week of plasmapheresis the basic caloric intake was increased to about 70 per kilo by the addition of more syrup and cod liver oil. During this week, following a blood exchange through the femoral vessels, a gas infection developed in the thigh and groin with necrosis and crepitation. Debridement of the area was performed under anesthesia. *B. welchii* was cultured from the wound. Fortunately the entire diet was consumed daily and evidence of intoxication was slight, but plasmapheresis was necessarily discontinued for several days. This accounts for the low protein output during the 11th period which was one of stomach feeding. Recovery was rapid and complete.

At the close of the 15th period, the dog became quite ill. The plasma protein level just before this illness was 4.0 per cent. 2 days later in spite of 36 per cent food consumption there was a marked drop in plasma protein concentration to 3.4 per cent. The next morning even with a 40 per cent food consumption of that day's ration, the plasma protein level had fallen to 3.1 per cent. This dangerous condition was tided over by several blood transfusions—a total of 360 ml. of whole blood and 400 ml. of 10 per cent glucose. The dog refused food, showed generalized edema and a tender fluctuant area in the neck. Two abscesses in the neck were drained. A small amount of liver and salmon was eaten and the dog rapidly recovered. Thus period 16 covers 13 days but is recorded as a 7 day period, the proper corrections being made. The basal diet was eaten the last 8 days of this period. The calories in the diet were again raised by syrup and cottonseed oil to about 80 per kilo, the protein intake remaining constant. A bleeding was done on the last day of the 16th period of sufficient size to reduce the plasma protein level which had climbed to 4.52 gm. per cent to 3.92 per cent. Periods 17 to 19 were satisfactory. Fecal nitrogen loss was not determined but was figured

as 1 gm. per day which is too high and increases the figures for negative nitrogen balance. The non-protein nitrogen of the plasma ranged from 11. to 26. mg. per cent being highest during the periods of supplemental feeding. In the middle of the 20th period, the dog became sick. The plasma protein level dropped to 2.88 per cent and the neck, jowls, chest, and forelegs became markedly edematous.

TABLE 6

Summary of Dicts and Potency Ratios

Dietary factors	D/P or potency ratio					
	From this paper					From previous papers
	Dog 33-324	33-333	34-53	34-152	33-11	
Beef serum						2.6
Salmon bread*						4.2
Bran				4.3		
Potato-bran basal ration	5.5	4.8	4.1±			
Rice polishings						(2.7)(4.2)(4.4)(4.5)
Gizzard				5.3		
Kidney basal ration						
Rice basal ration						5.3
Lactalbumin		5.4		5.3	5.3	
Skeletal muscle						
Egg white						5.5
Irish potato						5.7
Liver						5.8
Soy bean				6.1		
Liver residue				6.4		
Beef heart			4.6+	7.1		(6.5)(6.8)
Liver extract						
Casein						7.4
Spleen						8.0
Brain						8.6
Stomach				10 2		10.0
Salmon	13.6				11 8	
Pancreas						
Kidney						15.2
						19 0
						20 8

* 60 per cent of protein derived from wheat.

The neck was firm and tender. Two transfusions totaling 245 ml. of whole blood were given. Death occurred on the 4th day of illness. Autopsy revealed marked hemorrhagic edema of the neck and chest, sections of which showed numerous polymorphonuclear leucocytes, masses of bacteria, and edema. There were extensive deposits of iron in the form of pigment in the spleen, liver, lymph nodes, and bone marrow.

Table 6 shows all the *potency ratios* of the basal diets and supplemental feedings for comparison. The *potency ratio* means the number of grams of protein which must be fed to regenerate 1 gm. of plasma protein in a depleted standard dog.

DISCUSSION

Fasting causes a peculiar reaction in these dogs. If fasting (with or without dextrose by mouth) precedes the plasmapheresis it exhausts almost completely the reserve store of plasma protein building materials. When fasting periods are introduced after the dog is down to its basal output of plasma protein on the given basal ration, the fasting reaction is very constant. The fasting dog will then put out 4 to 6 gm. plasma protein per week and cannot be forced to produce more, whereas if placed back on a standard ration it will pour out new protein into the plasma promptly and in amounts determined by the character and amount of the basal diet. There is urgent need for this plasma protein but during fasting the dog can produce only this trifling amount. The body proteins cannot contribute significantly to the plasma proteins in an emergency but the plasma proteins do contribute readily to the body proteins and in a fasting emergency we can keep the dog in nitrogen equilibrium by means of plasma given intravenously (15). There would seem to be a difference between this *reserve store of protein* building material and the other body proteins for one will contribute promptly to form new plasma protein and the other will not contribute any significant amount. We have pointed out that the reaction in fasting is very different from hemoglobin regeneration in anemia in which the fasting dog can produce 25 to 35 gm. new hemoglobin as the result of a week's fast. Likewise the fasting dog (dextrose by mouth) can regenerate a liver injury due to chloroform and we may say that with a liver weighing 400 gm. showing a 50 per cent necrosis we can follow the regeneration to completion during a 2 weeks dextrose period. This means the regeneration of 200 gm. of liver tissue. Evidently the plasma proteins, once the reserve store is exhausted, are regenerated almost wholly from food materials coming in from the gastro-intestinal tract.

Infection (Table 5) may seriously interfere with the production of plasma protein. One may choose to infer that absorption is disturbed

and is wholly responsible for this change but we cannot agree. We prefer to think of the *intoxication* as in part responsible for this disturbance due to its effect on internal metabolism. It is well known that with abscess and intoxication there is a very large increase in urinary nitrogen which surely indicates body protein injury. There may be a sharp drop in the blood plasma protein level (Clinical History, Dog 33-324) during the acute intoxication. This may be due largely to change in capillary permeability and resultant edema but it is a vicious circle as the drop in plasma protein concentration favors further transudation and edema. One may argue that the intoxication causes the drop in plasma protein which is responsible for the edema or *vice versa*. Plasmapheresis definitely lowers the dog's resistance to infection particularly when a heavy protein intake necessitates very large blood exchanges. Furthermore when plasma protein depletion is carried to the edema producing level (3.2 to 3.5 gm. per cent) one notes at once a disturbed clinical state, the dog is less lively, and there is loss of appetite.

Soy bean is a food factor (Tables 1 and 3) which deserves special mention. It causes an extraordinary reaction which is obvious within 24 hours—a reaction even more prompt than noted with most animal proteins. There is little carry over into the control week—in other words it is promptly and quite completely utilized during the feeding period, in contrast to many grain proteins which show a slow utilization with the greater amount of the new formed plasma protein appearing in the week following the feeding.

When one notes that plant proteins in the diet favor the production of globulin and that this reaction is slower than that recorded for albumin, one cannot avoid the thought that to manufacture the larger globulin molecule requires more time which may be a factor in the reaction. Furthermore plant proteins as a rule favor the production of globulin in these experiments but soy bean like animal proteins favors the production of albumin. The potency ratio of soy bean meal is between 5. and 7. which places it in a class with liver. This food material deserves study in human conditions associated with hypoproteinemia and might be of value in the diets utilized in renal disease.

It might be inferred that the human with diseased kidneys should be fed diets which favor globulin production (plant proteins because

this plasma globulin might not escape as readily as the smaller albumin molecules through the diseased kidney. Although the globulins do not have as much influence on osmotic equilibrium yet the small effect might be just enough to prevent tissue edema which is so disturbing to the patients.

SUMMARY

When blood plasma proteins are depleted by bleeding, with return of washed red cells (plasmapheresis), it is possible to bring the dog to a steady state of low plasma protein in the circulation and a uniform plasma protein production on a basal diet. These dogs become test subjects by which the potency of various diet factors for plasma protein regeneration can be measured.

Plant and grain proteins are quite well utilized to form new plasma protein in these test dogs but *soy bean* meal probably should be rated at the head of this list. It is utilized with unexpected promptness and favors the production of albumin in contrast to other plant proteins which distinctly favor globulin production.

Long plasmapheresis periods on basal rations rich in grain proteins lower the resistance of these animals to infection.

Spleen, brain, and stomach when fed with the basal diet in these test dogs show less favorable potency ratios—10.2, 11.8, and 13.6 respectively. This means the grams of tissue protein which must be fed to produce 1 gm. of new plasma protein.

Fasting periods indicate that the dog can contribute only 4 to 6 gm. of plasma protein each week—an insignificant contribution presumably derived from the host's tissue proteins.

Infection and intoxication disturb the plasma protein production of these standardized dogs and may reduce the output of plasma proteins to very low levels in spite of considerable food intake. There may be a very sharp drop in the plasma protein level during the first day of intoxication (Dog 33-324).

Some of these observations may be of value in a study of clinical conditions associated with hypoproteinemia.

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THE VISCERAL LESIONS PRODUCED IN MICE BY THE SALIVARY GLAND VIRUS OF MICE*

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PLATES 24 TO 26

(Received for publication, December 4, 1935)

Intranuclear inclusions have been described in the cells of the salivary glands of many species and, in several instances, have been shown to be due to a filterable virus. In all these species the virus has been looked upon as a harmless one which, although responsible for the salivary gland lesion, produces no clinical evidence of disease in the natural infection.

Experimentally, when an emulsion of salivary gland tissue of adult guinea pigs, mice, hamsters, or rats is injected into the same species, characteristic lesions in the salivary gland are produced whether the inoculation is intraperitoneal, subcutaneous, or intraglandular.[1,2,3] The production of lesions in other organs except at the site of inoculation has not been described. When Cole and Kuttner[1] injected the guinea pig salivary gland virus into the tongue, testicle, or lung of the guinea pig, they produced a local subacute inflammation with intranuclear inclusion bodies in these organs. Kuttner[2] and Kuttner and Wang[3] were also able to produce with the virus specific for the species a meningitis, fatal in some instances, in guinea pigs, mice, hamsters, and rats. Generalized visceral lesions were not produced in any of these experiments and only in the case of the intracerebral injections was a fatal disease produced in any species.

We have produced extensive visceral lesions in mice of the Buffalo

* These investigations were carried out with the aid of a grant for research in science made to Washington University by the Rockefeller Foundation.

[1] Cole, R., and Kuttner, A. G., *J. Exp. Med.*, 1926, 44, 855.

[2] Kuttner, A. G., *J. Exp. Med.*, 1927, 46, 935.

[3] Kuttner, A. G., and Wang, S., *J. Exp. Med.*, 1934, 60, 775.

strain with the mouse salivary gland virus by intraperitoneal and intracerebral injections. Also after subcutaneous inoculations focal areas of inflammation with intranuclear inclusion bodies were occasionally found in the pancreas in addition to the usual salivary gland lesion. In a series of four experiments, an infection fatal in 4 to 7 days with extensive visceral lesions was produced in more than half of the animals by intraperitoneal inoculation of the virus. Fatal infections were produced less often by intracerebral injections.

Methods

The material for the inoculations was prepared as follows: Our stock Swiss mice have shown intranuclear salivary gland inclusions in a high percentage of the adult animals examined and, on the other hand, mice from the Buffalo strain have failed to show similar inclusions either in the adult or the young animals examined. Therefore the salivary glands of several adult Swiss mice were used as the source of the virus and young mice of the Buffalo strain approximately 3 weeks of age were used for the remainder of the experiments. Since neither the adult nor the young animals which we examined from the Buffalo strain have shown inclusions it seems safe to assume that these young animals were not infected.

The salivary glands of several adult Swiss mice were emulsified in broth (approximately one gland to ¾ cc. broth) and the emulsion centrifuged. With ¼ cc. of the supernatant fluid young mice of the Buffalo strain were injected subcutaneously. After 2 weeks, the salivary glands of these animals prepared in the same way were used for a series of experiments and also to inoculate subcutaneously another group of young Buffalo mice to carry on the virus. In each instance the amount of solution injected either subcutaneously or intraperitoneally was ¼ cc. For intracerebral inoculations 0.02 cc. of solution was used. For both the intraperitoneal and intracerebral inoculations, the centrifuged solution was also passed through a Berkefeld V filter to avoid transferring the paratyphoid infection which was endemic among the stock mice.

RESULTS

In four experiments forty-two young mice of the Buffalo strain were inoculated intraperitoneally. Twenty-eight of these animals died or were killed when moribund between the 4th and 7th day. Some of the animals which survived were killed and autopsied later. In the animals autopsied between the 4th and 7th day after inoculation, the most extensive lesions were in the liver, spleen, adrenals, lymph nodes, and the subperitoneal connective tissue and fat. Less extensive changes were found in the lungs, kidneys, intestines, and pancreas.

Lesions were not found in the salivary glands of animals autopsied before the 8th day but after the 8th day intranuclear inclusion bodies were found in some of the acinar cells of the salivary glands whenever they were examined.

In the gross, the liver was enlarged, very pale, and at times showed small areas of hemorrhage. The spleen was somewhat enlarged and friable. The lungs were more congested than normal and sometimes showed small hemorrhagic areas. There was no peritoneal exudate and the peritoneal lining was smooth. Cultures from the peritoneal cavity were negative. The serous surface of the intestines was smooth but individual intestinal loops were occasionally very red and the mucosa in these areas was intensely congested.

Microscopically, the liver lesion varied in intensity. In many liver cells, but especially in those nearest the portal zones, the nuclei were hypertrophied and contained large eosinophilic intranuclear inclusions. Most of these inclusions were larger than those usually found in the mouse salivary gland. However, the inclusions and also the nuclei varied in size. In the smaller nuclei which contained small inclusions the chromatin was not so distinctly marginated as it was when the nucleus and its inclusion were larger. With hematoxylin and eosin the staining of the inclusions varied but they were always less basophilic than normal nuclear chromatin. Occasionally intranuclear inclusions were seen in Kupffer cells, in connective tissue cells about the large bile ducts, and in the multiple nuclei of megalokaryocytes in liver sinusoids. Liver cells with two nuclei each containing an inclusion were also found.

The inclusions in the liver cells were round or oval, of various sizes, and slightly irregular in outline. In the well preserved cells they seemed to be coarsely granular or made up of masses of material molded together. In some nuclei, the irregularly outlined inclusions seemed lobulated and in other nuclei two or three distinct masses of eosinophilic material were present. The large number of inclusions produced in a short time seems to offer an excellent opportunity for further detailed study of the structure of these bodies. The cytoplasm of the liver cells containing inclusions was finely granular and stained purplish red with hematoxylin and eosin. No basophilic cytoplasmic inclusions occurred.

Necrosis of the liver cells occurred, involving single cells, only a few cells, or a large section of a lobule. Large areas of necrosis were usually midzonal in position and were often associated with hemorrhage (Fig. 1). Polymorphonuclear leukocytes accumulated about the liver cells which contained inclusions even before necrosis of the cells occurred (Fig. 2), but they were especially numerous about necrotic cells or groups of cells. The cells of this exudate, which consisted principally of polymorphonuclear leukocytes but also some mononuclear cells, later became necrotic also. On the 5th day after inoculation, many cells with intranuclear inclusion bodies were seen and areas of necrosis with hemorrhage were present. However, these changes were at times found as early as the 3rd day. On

the other hand, in animals autopsied on the 6th or 7th day, there were, as a rule, fewer inclusions but larger groups of liver cells were necrotic and the inclusions were found chiefly in cells about the edge of the necrotic zones. There were also groups of large, pale staining liver cells without inclusions, usually near the central veins, which had vacuolated cytoplasm and sometimes contained fat droplets. In a few animals autopsied later small areas remained where necrotic liver cells seemed to have been replaced by a loose stroma infiltrated with wandering cells. All the intranuclear inclusions had disappeared.

The lesion in the spleen also varied in degree. In animals dying on the 4th and 5th day the spleen was often almost entirely replaced by necrosis and hemorrhage with a few large mononuclear cells containing large intranuclear inclusions still visible where the splenic tissue was not entirely necrotic (Fig. 5). In other animals with less advanced lesions, the hemorrhage and necrosis were most marked about the Malpighian bodies, and there were innumerable large mononuclear cells with pale blue, finely granular cytoplasm and a large nucleus containing a large oval, or round, or greatly elongated inclusion body. The shape of the inclusion usually corresponded to that of the nucleus in which it occurred. There were many polymorphonuclear leukocytes where necrosis had taken place. In spleens in which necrosis had not occurred, there was a marked proliferation of large mononuclear cells, probably reticulum cells. These were chiefly at the margin of the Malpighian bodies and it was in these cells that the inclusions were most conspicuous. Multinucleated cells with an inclusion in each nucleus were occasionally seen.

Lesions were always present in the adrenals. In some animals only a few groups of cells in the cortex contained intranuclear inclusions. These cells became necrotic and surrounded by polymorphonuclear leukocytes. In the most extreme lesions there were extensive necrosis and hemorrhage in the inner half of the cortex (Fig. 3) and almost every remaining cell of the cortex had a hypertrophied nucleus containing an inclusion (Fig. 4). In some animals which survived and were killed later there were found areas of degeneration in the cortex containing a few mononuclear phagocytic cells.

In the kidney, inclusions were not found in the cells of the tubules, but a few glomeruli contained one or more large cells with an intranuclear inclusion. These cells usually occurred within the tuft and it was impossible to determine whether they were endothelial cells of the capillaries, or epithelium covering loops of the tuft.

Sections made through loops of intestine congested in the gross showed an extremely hyperemic mucosa. A few intranuclear inclusions were found in connective tissue cells of the mucosa and in epithelial cells deep in mucosal glands.

In lobules of fat tissue and loose connective tissue of the omentum and of the retroperitoneal tissue about the pancreas and kidney there was an inflammatory process with an increase in mononuclear cells and proliferation of fibroblasts. The capillaries were engorged and in addition there seemed to be newly formed capillaries. Many of the large mononuclear wandering cells and fibroblasts

contained intranuclear inclusions. They were also seen a few times in the swollen endothelium of capillaries. Some polymorphonuclear leukocytes were present especially where necrosis of cells occurred. Occasionally cells of the serous lining of the peritoneal cavity were enlarged and contained intranuclear inclusions.

Small lymph nodes near the duodenal end of the pancreas, near the kidney, and also at the hilum of the lungs showed foci of necrosis and hemorrhage just inside the peripheral sinus. Large mononuclear cells with intranuclear inclusions were seen in or near these foci.

In two instances sections of the ovary were made and in each case intranuclear inclusions were found in some of the cells of the theca interna.

Pathological changes were found in the lung in more than half of the animals that died. These consisted of focal areas of cellular infiltration about small vessels and in the alveolar walls with thickening of the latter. The infiltrating cells consisted of both polymorphonuclear leukocytes and mononuclear cells. Cells with intranuclear inclusions were frequently found in these foci. Whether a cell containing an inclusion lined a capillary or was a wandering cell could not always be determined. At times, however, the inclusions were definitely in cells lining alveoli. Intranuclear inclusions were also found in connective tissue cells about large bronchi and where they were present there was also an inflammatory exudate composed of polymorphonuclear and mononuclear cells. Rather large areas of alveolar hemorrhage occurred in some lungs in which the intranuclear inclusions were numerous.

In the pancreas lesions were found in more than half of the animals autopsied between the 4th and 7th day after intraperitoneal inoculations. There were intranuclear inclusions in a few acinar cells and there were small foci of wandering cells, both polymorphonuclear and mononuclear cells. In one instance, intranuclear inclusions were found in the cells of Brunner's glands in the duodenum immediately adjacent to the pancreas. The inclusions in the pancreatic cells were usually smaller than those in the other viscera. They resembled more those found in the salivary gland. It is of interest that the pancreatic lesions were less intense than those in other abdominal organs after intraperitoneal inoculations and yet pancreatic lesions were the only ones found outside the salivary glands 2 weeks after subcutaneous inoculation.

Cellular inclusions were not found in the salivary gland of mice dying between the 4th and the 7th day after intraperitoneal injection. But they were found in the salivary gland of all animals that survived and were autopsied after the 8th day. They were almost always found in acinar cells of the serous acini in a part of the gland which contains both mucous and serous glands, rarely in duct cells. In addition small basophilic cytoplasmic bodies were occasionally present in the affected cell. The inclusions in mice were more acidophilic than those found in the salivary glands of infants. The cells containing these inclusions and their nuclei were hypertrophied but usually not to such a marked degree as seen in the guinea pig and in the human salivary gland. However, both the size of the inclusions and the degree of hypertrophy of the cells varied considerably. Our observa-

tions in regard to the character of the inclusions and the cells containing them agree exactly with the descriptions given by Kuttner and Wang.[3] Some degree of lymphocytic infiltration was always present in the glands in which the inclusions were found but not always near the cells which contained them. The cellular infiltration was usually more extensive and diffuse than that seen in the salivary glands of the stock Swiss mice which showed inclusions.

In spite of the fact that such extensive and fatal lesions were produced in the liver and spleen, we were unable to pass the virus in series with material from these organs. In a few instances a small number of intranuclear inclusions were found in the spleen of an animal receiving this liver and spleen material but extensive lesions were never produced and the animals did not become sick. However animals of this group which were autopsied 2 weeks after inoculation showed intranuclear inclusions and an infiltration of mononuclear cells in the salivary glands.

Following intracerebral inoculation of mice of the Buffalo strain extensive generalized lesions were at times produced with the same distribution and character as those produced by intraperitoneal inoculation. These animals died between the 3rd and 7th day. At other times, perhaps because the solution of virus was less concentrated, the animals survived 7 days and when autopsied showed no lesion except a slight mononuclear meningeal exudate with a few intranuclear inclusions in the cells of the exudate.

Mice injected subcutaneously did not become sick. Those autopsied after 2 weeks showed the characteristic salivary gland lesions. Organs other than the salivary glands from these animals were not examined as a routine, but in the few instances in which they were examined inclusions in organs other than the salivary glands were found three times. These were in the acinar cells of the pancreas.

DISCUSSION

The failure of others to produce generalized visceral lesions in mice with the salivary gland virus may have been due to the fact that they have used smaller amounts of the virus for inoculation, but there is also the possibility that there is some difference in the susceptibility of different strains of mice. We have not been able to produce extensive lesions so readily in black mice of the C57 strain, while we have

some indication that the Swiss mice are even more susceptible than those of the Buffalo strain.

The inability to transmit the virus in series with liver and spleen in spite of the innumerable intranuclear inclusions and extensive damage produced in these organs is puzzling. Cole and Kuttner[1] reported the same difficulty in transferring the salivary gland virus of the guinea pig with material other than the salivary gland but they had not produced lesions of such intensity in the organs which they had inoculated locally and then attempted to use for passing the virus. Kuttner[2] believed that the guinea pig virus lost virulence after repeated transfers even when a salivary gland emulsion was used to pass the virus. In our experiments we were able to produce extensive visceral lesions by intraperitoneal inoculation of salivary gland emulsions after the sixth transfer by the subcutaneous route. No decrease in virulence was detected.

The salivary gland, although it becomes the site of a persistent infection in mice which survive, does not show inclusions or cellular infiltration at the time when the mice inoculated intraperitoneally die, showing many intranuclear inclusions in the abdominal organs, lungs, and lymph nodes. Whether the virus can be passed by salivary gland emulsions at this early period has not been determined.

The experimental production of visceral lesions with mouse salivary gland virus, possessing a low natural pathogenicity, suggests the possibility that the natural infection may at times become generalized, perhaps in especially susceptible strains or individuals. By analogy the suspicion is strengthened that the generalized lesions seen in infants with intranuclear inclusions similar to those occurring in the salivary gland are due to a generalization of the salivary gland virus. Kuttner and Wang,[3] in their review of the literature, call attention to the fact that intranuclear inclusions have not been described in the salivary glands of infants when they have been found in various other organs. For this reason, they raise the question as to the identity of the virus concerned in the two groups of cases. However, the failure to describe salivary gland lesions in infants in association with visceral lesions containing this type of inclusion may be due in large part to the failure to make routine examinations of the salivary gland. In our material from the St. Louis Children's Hospital and the St. Louis

Isolation Hospital we have found in eight infants intranuclear inclusions in the salivary glands together with similar inclusions in other organs. The salivary gland was examined in only one of twenty-nine other infants and young children who showed such inclusions in other organs.

CONCLUSIONS

Extensive visceral lesions containing intranuclear inclusions have been produced in mice by intraperitoneal and intracerebral inoculations of the homologous salivary gland virus. Rarely small pancreatic lesions containing inclusions have been encountered 2 weeks after subcutaneous inoculation.

Many of the animals injected intraperitoneally died between the 4th and 7th day after inoculation.

In spite of the extensive lesions produced in the liver and spleen, the virus could not be transferred with an emulsion of these organs.

EXPLANATION OF PLATES

All sections have been stained with hematoxylin and eosin.

PLATE 24

FIG. 1. Low power of mouse liver, showing destructive lesions caused by salivary gland virus. The black areas represent necrosis of liver cells with inflammatory exudate. ×30.

PLATE 25

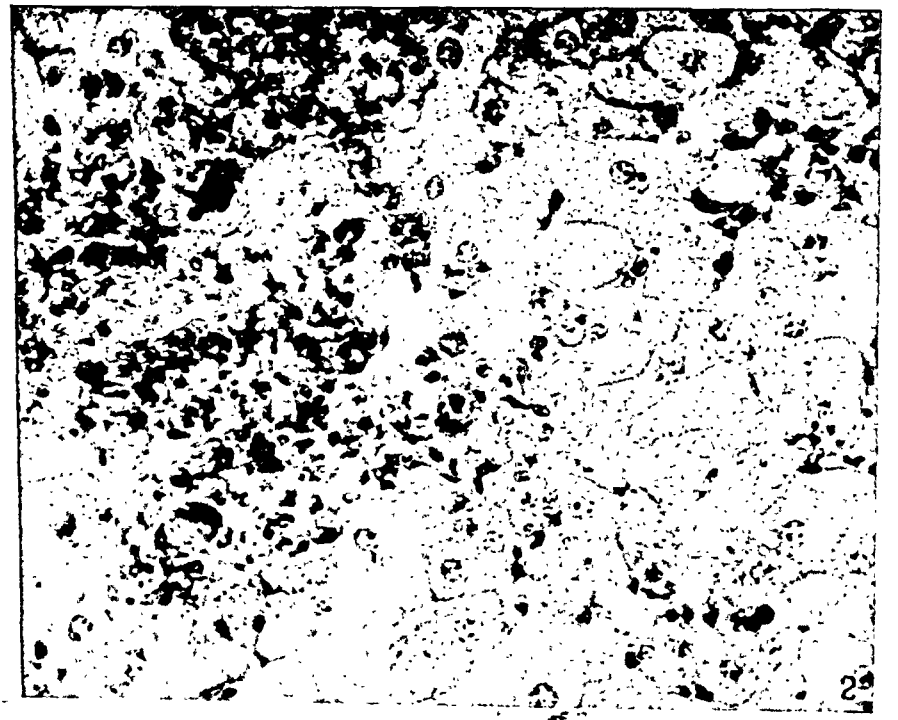
FIG. 2. High power of liver lesion with necrosis and cellular infiltration. An intranuclear inclusion can be seen in a liver cell near the center of the field. ×300.

FIG. 3. Adrenal gland showing areas of necrosis and hemorrhage in the inner cortical layer. ×20.

PLATE 26

FIG. 4. High power of adrenal with numerous intranuclear inclusions and area of necrosis and hemorrhage in upper left hand corner. ×300.

FIG. 5. Area from a section of mouse spleen. The black masses are collections of fibrin about necrotic cells. Many inclusion bodies are present in the nuclei of the large mononuclear cells. ×300.



ACTIVE IMMUNIZATION OF GUINEA PIGS WITH THE VIRUS OF EQUINE ENCEPHALOMYELITIS

I. Quantitative Experiments with Various Preparations of Active Virus

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(Received for publication, November 15, 1935)

The object of this paper is to describe attempts at determining the minimal quantity of the virus of equine encephalomyelitis needed for producing resistance to experimental infection of guinea pigs by way of the nose, skin, and brain. With this known, a basis has been constructed for comparing the immunizing capacity of virus preparations. The Eastern and Western strains were employed in this study in three forms: adsorbed on alumina gel; precipitated by tannin; and chemically untreated, merely passaged through a series of mice.

Evidence has been brought forward by several investigators (1) to show that active virus of equine encephalomyelitis immunizes horses and guinea pigs against experimental infection. Shahan and Giltner (2) report that protection can be secured by means of formolized infected tissue which, they believe, contains inactivated virus. Others, on the contrary, state that inactive material is apparently ineffective (Howitt, 1; Rosenbusch, 1).

The experiments on immunity with active, untreated virus passaged through a large series of white mice will be described first.[1]

Active, Untreated Virus as Immunizing Substance

It is known that certain viruses, after repeated animal passage, are reduced or modified in their infectivity for the original host and hence can be used for immunization (3). Besides the pox diseases, other maladies, as, e.g., rabies (3), horse sickness (4), yellow fever (5), and psittacosis (6), have been successfully prevented by means of adapted material. Traub and TenBroeck (1) have recently reported

[1] Operations on animals were made with the aid of ether anesthesia.

the immunization of guinea pigs and horses with pigeon-modified, equine encephalomyelitis virus (7).

TABLE I

The Comparative Susceptibility of Mice and Guinea Pigs to Intracerebral Injection of the Same Quantities of Mouse Passaged Virus

Virus dilution	Result	
	Mice	Guinea pigs
10^{-5}	6/6	4/4
10^{-6}	6/6	4/4
10^{-7}	1/6	1/4
10^{-8}	0/6	0/4
10^{-9}	0/6	0/4

Quantity = 0.03 cc.

Numerators represent number of animals that succumbed to infection; denominators, number of animals employed in the test.

TABLE II

Guinea Pig Brain Virus Injected Subcutaneously in Guinea Pigs

Time after injection	Virus dilution									
	10^{-2}	10^{-3}	10^{-4}	10^{-4}	10^{-5}	10^{-5}	10^{-6}	10^{-6}	10^{-7}	10^{-7}
days	°F.	°F.	°F.	°F.	°F.	°F.	°F.	°F.	°F.	°F.
1	101.4	102.5	101.6	102.4	101.8	102.4	102.0	102.0	101.4	101.4
2	104.5	104.6	101.2	105.4	105.0	105.3	101.8	105.4	102.7	102.2
3	100.8	102.4	101.8	102.4	101.6	102.3	102.1	D	101.9	102.8
4	102.7	105.8	101.8	104.6	102.4	102.4	102.3		103.0	102.8
5	D	100.3	100.6	D	105.4	101.6	101.6		101.7	101.8
6		D	102.4		D	102.1	101.1		102.2	103.0
7			101.6			102.8	102.0		102.0	104.6
8 to			102.3			103.0	102.9		102.9	D
21			S			S	S		S	

Weight of animals = 275 to 325 gm.

D = died of experimental encephalitis.

S = survived and apparently normal.

Source of Virus.—The Eastern and Western strains, ultimately derived from affected horses and propagated in guinea pigs, were transferred to the brain of mice. The virus was passed serially in this way from mouse to mouse until the 40th, or a still further passage when modification of the virus was revealed by its

diminished pathogenicity after injection in the subcutis of guinea pigs. This was not true as concerns injections into the brain. To illustrate, the same quantity of mouse brain virus as before (0.03 cc.) was lethal intracerebrally in mice and guinea pigs in dilutions of 10^{-4} to 10^{-7} inclusive. It was now, however, not disease-producing when 1 cc. of these dilutions was injected subcutaneously in guinea pigs. By contrast, guinea pig brain virus, before mouse passage, was active in guinea pigs when given subcutaneously, regularly in dilutions up to 10^{-3} and occasionally up to 10^{-7} (see Tables I, II, and III which summarize the results of such titrations and are examples of repeated observations).

Preparation of Material.—For use as antigen the brains of mice which succumbed to mouse passage virus were removed aseptically and ground to a 10 per cent sus-

TABLE III

(For Comparison with Table II)

The Results of Mouse Adapted Virus Injected Subcutaneously in Guinea Pigs Weighing 275 to 350 Gm.

Time after injection	Virus dilution													
	10^{-2}	10^{-3}	10^{-4}	10^{-4}	10^{-5}	10^{-5}	10^{-6}	10^{-6}	10^{-7}	10^{-7}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
days	°F.	°F.	°F.	°F.	°F.	°F.	°F.	°F.	°F.	°F.	°F.	°F.	°F.	°F.
1	102.8	102.6	103.4	104.4	102.6	103.0	103.9	101.8	103.5	103.7	0/4	0/4	0/4	0/4
2	105.5	105.8	104.6	104.4	103.3	103.5	103.4	103.8	103.0	103.2	4/4	2/4	0/4	0/4
3	102.4	104.4	102.5	103.6	102.0	103.0	103.0	101.6	101.5	101.3		4/4	3/4	1/4
4	101.0	102.5	101.2	102.6	101.5	102.0	102.6	101.2	102.0	101.4			3/4	1/4
5	103.8	103.7	103.4	104.1	103.6	103.7	103.2	104.0	103.3	103.9			3/4	2/4
6 to 21	S	S	S	S	S	S	S	S	S	S			4/4	2/4

At right of double line is shown a simultaneous titration of virus by intracerebral inoculation of mice; the numerator represents number of mice that died of the experimental disease; the denominator, the number of animals employed in the test.

pension in broth. The suspension was spun in an angle centrifuge at 3,000 R.P.M. for 15 minutes and the required dilutions were prepared from the supernatant fluid.

This constituted fresh material, but it was often desired to inject animals with known quantities of virus and to repeat the injection at weekly intervals; hence the material had to be maintained in undiminished concentration. Two methods of preservation were employed: merthiolate and desiccation. Of the two, the latter was preferable since the virus content did not diminish as rapidly over long periods of time.

A 2 per cent suspension of infected mouse brains in Tyrode's solution, pH 7.6, containing merthiolate 1:5,000 was made. The suspension was filtered through 8 ply sterile gauze, diluted with three volumes of sterile, distilled water, and stored

in the cold. The material, which contained 300,000 mouse infective units (hereafter called m.i.u.)[2] per cc. immediately after its preparation, was found after storage for 4½ months to contain 3,000 m.i.u. per cc. of virus. Desiccated material was obtained as follows: Mouse brains removed from animals before death were ground to a 10 per cent suspension in Tyrode's solution, pH 7.6, or in 10 per cent normal rabbit serum diluted with Tyrode's solution. After centrifugation at 4,000 R.P.M. in an angle machine for 20 minutes, the supernatant fluid was removed and tubed in 1½ cc. quantities and dried *in vacuo* in the cold over sulfuric acid. Dried virus, after storage for 12 weeks, still contained the original amount of the active agent (usually 3×10^5 to 3×10^6 m.i.u. per cc. of resuspended material). The effect of longer storage on dried virus has still to be determined.

The two strains, Eastern and Western, prepared separately or mixed in the same specimen, exhibited no noticeable difference in immunizing power.

Dosage.—All virus samples were titrated before use by mouse intracerebral inoculation. In this way the number of m.i.u. of virus was determined. For preventive inoculation of guinea pigs 1 cc. was injected subcutaneously and, when indicated, repeated at 7 to 13 day intervals. Control guinea pigs and mice were included in each experiment to measure the activity of varying dilutions of virus by means of inoculation intracerebrally, or subcutaneously, or by instillation intranasally (see Tables I to III for a pattern of such titrations).

Test for Resistance.—Although guinea pigs were immunized with mouse passage virus, they received as test dose for induced resistance guinea pig brain passage virus, as did all animals throughout this study.

An intracerebral test was found to be more drastic than an intranasal or a subcutaneous one. To illustrate, guinea pigs received subdurally 0.12 cc., subcutaneously, 1 cc., or intranasally, 0.1 cc. of virus suspension. With the volumes used, normal guinea pigs reacted with characteristic lethal encephalitis in limiting dilutions of 10^{-6} or even 10^{-7} after intracerebral inoculation; and the same happened with 10^{-3} on subcutaneous injection, and with dilutions of 1:100 to 1:200 after intranasal instillation. Therefore, to determine induced resistance, 10^3 to 10^4 M.L.D. were introduced into the brain, whereas in the skin or nose, lightly centrifuged, 10 per cent fresh virus suspension was employed, representing 1 to 10 M.L.D.

Effect of Storage.—The virus employed as immunizing agent was still effective after it had been stored in the cold for 3 months, at least.

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[2] A mouse infective unit (m.i.u.) is defined as the minimal amount of virus which, after intracerebral inoculation of mice, induces lethal encephalitis. If, for example, the highest active dilution is 10^{-8} (all dilutions are in terms of weight of infective tissue), then 10^{-5} represents 10^3 or 1,000 m.i.u. Since mice receive only 0.03 cc. intracerebrally, 1 cc. contains about 30 times as many units. Thus, 1 cc. of a 10^{-5} dilution should have approximately 30,000 m.i.u.

Immunization with Mouse Passage Virus.—We first sought to find the minimal amounts and number of doses necessary for active immunization against the intracerebral test dose. Table IV summarizes the experiments.

TABLE IV

The Relation of Amount of Mouse Passage Virus and Number of Doses to the Production of Resistance against Intracerebral Test Inoculation

No. of animals injected	No. of weekly injections	M.i.u. in each dose	Animals dying of infection during immunizing period	Animals showing fever of 104° or above	Animals showing virus in blood	Immunity test (4 to 6 wks. later)		
						M.L.D.	Died	Survived
7	1	3×10^5	4	7	7	10^3–10^4	1	2
7	1	3×10^7	2	7	6	10^3–10^4	0	5
8	1	3×10^6	1	7	7	10^3–10^4	1	6
8	1	3×10^5	0	2	2	10^3–10^4	0	8
2	2	3×10^5	0	1	1	10^2–10^3	0	2
12	2	3×10^5	0	4	3	10^3–10^4	2	10
3	3	3×10^5	0	2	1	10^2–10^3	0	3
7	3	3×10^5	0	3	2	10^3–10^4	0	7
9	1	3×10^4	0	2	0	10^2–10^3	6	3
7	1	3×10^4	0	2	0	10^3–10^4	4	3
2	2	3×10^4	0	0	0	10^2–10^3	2	0
20	2	3×10^4	0	4	1	10^3–10^4	10	10
2	3	3×10^4	0	0	0	10^2–10^3	0	2
41	3	3×10^4	0	3	0	10^3–10^4	0	41
6	1	3×10^3	0	0	0	10^2–10^3	6	0
11	1	3×10^3	0	0	0	10^3–10^4	7	4
9	2	3×10^3	0	0	0	10^3–10^4	4	5
19	3	3×10^3	0	0	0	10^3–10^4	0	19
7	1	3×10^2	0	0	0	10^2–10^3	7	0
11	1	3×10^2	0	0	0	10^3–10^4	11	0
9	2	3×10^2	0	0	0	10^3–10^4	9	0
8	3	3×10^2	0	0	0	10^3–10^4	6	2
9	1	3×10^1	0	0	0	10^3–10^4	9	0

Reference to the table shows that lessened resistance occurred in animals treated with amounts of virus smaller than 3×10^2 m.i.u., and even with this amount if it was given less than three times. Larger quantities, *i.e.*, more than 3×10^4 m.i.u., injected less than three times, were also effective in developing immunity but the danger then arose of increasing correspondingly the incidence of fever, circu-

lating virus,[3] and death during the period of immunization. In other words, it was essential that the preventive dose should confer protection with the use of the least possible amount of virus, thus minimizing febrile reactions and blood infection. This dose has been determined as 3×10^3 to 3×10^4 m.i.u. injected subcutaneously three times at weekly intervals. It will be noted that absence of fever during the period of immunization does not necessarily foretell a diminution in the degree of induced protection.

Immunization against Nasal and Subcutaneous Infection.—The next series of experiments (Table V) reveals that protection against nasal and subcutaneous test injections can be secured by means of one or two subcutaneous injections, each containing 3×10^4 m.i.u. of virus.

At this point, the fact should be mentioned that normal mice and guinea pigs receiving virus nasally and resisting infection may be susceptible to a later nasal instillation. Again, animals injected subcutaneously once or twice with smaller amounts of active virus are refractory to the nasal test but may succumb to the disease after a later intracerebral inoculation. Apparently intranasal application of virus fails to increase the degree of resistance. This is in striking agreement with the results obtained by Jungeblut and Hazen (8) and by Flexner (9) in studies on experimental poliomyelitis in the monkey. As stated by Flexner, refractory monkeys which resist not only one but several courses of nasal instillations of poliomyelitis virus develop no indication of either active or passive immunity.

In summing up the series in which untreated mouse passage virus was used as immunizing agent, it appears that a sufficient amount of the material and its periodic inoculation are required to produce effective protection. The sufficient amount has been measured as 3×10^3 to 3×10^4 m.i.u.: one or two such doses given subcutaneously protect

[3] The usual procedure for detecting circulating virus is limited in application, since 0.03 cc. of blood derived from an affected guinea pig, usually during the height of fever, or from the 2nd to 5th day after the primary injection, is introduced into the brain of each of four or six mice for the specimen to be examined. While positive reactions are unequivocal, negative ones are less significant for only a small amount of blood is put to test. However, these tests were made with the purpose of comparing the results obtained in each series of animals treated with active virus.

guinea pigs against experimental subcutaneous or intranasal infection and three doses, given at weekly intervals, against intracerebral test inoculation of 10^3 to 10^4 lethal units. Further, fever occurred in only eleven of 126 animals given this dosage, and in only one instance was

TABLE V

Protection Test Given by Way of Nose and Subcutis

No. of subcutaneous injections of mouse virus	Strain and m.i.u. in each dose	Test for resistance given 14 to 21 days after last immunizing dose	Given by way of	Result*
1	Eastern = 3×10^4 Western = 3×10^3	1–5 M.L.D.	Subcutis	1/3
1	Eastern = 3×10^4 Western = 3×10^3	1–5 M.L.D.	Nose	2/3
1	Eastern = 3×10^4	1–5 M.L.D. on 2 occasions, 4 days apart	"	1/6
1	Eastern = 3×10^3	1–5 M.L.D. on 2 occasions, 5 days apart	"	3/7
1	Eastern = 3×10^4	1–5 M.L.D.	Subcutis	2/22
1	Eastern = 3×10^4	1–5 M.L.D.	Nose	1/11
2	Eastern = 3×10^4	1–5 M.L.D.	Subcutis	0/10
2	Eastern = 3×10^4	Tested on 2 occasions 4 days apart	Nose	1/6†

* Fractions defined in Table I; of thirty control guinea pigs used, twenty-seven died of characteristic encephalitis after nasal or subcutaneous injection of the test dose.

† These six guinea pigs withstood an intranasal test 16 days after the last preventive dose was inoculated but one of them died after all had received a second intranasal test 4 days later.

the febrile reaction accompanied by the presence of virus in the blood as determined by our method. The incidence of fever and of blood infection increases in those animals receiving larger amounts of virus—quantities in excess of what is needed for building up resistance. After storing, the longest period thus far being 3 months,—the im-

munizing agent still contained an adequate amount of virus for the production of uniform resistance to the usual intracerebral test. Finally, the protection acquired is durable, lasting throughout at least 3 months.

Adsorbed Virus as Immunizing Preparation

Aluminum hydroxide, Type C, first employed by Willstaetter (10) to purify enzymes, was chosen as the agent for adsorption (11). It was shown in a preliminary report (12) that the material adsorbed the encephalomyelitis virus and then could induce active protection in guinea pigs. The subject has since been more extensively elaborated and the results of this work are now presented.

Mouse rather than guinea pig passage virus was used in preparing vaccines for guinea pigs. It has already been pointed out (see also Tables II and III) that the mouse passage virus consistently displays a lesser degree of infectivity following subcutaneous injection of guinea pigs. In general, the methods employed with adsorbed virus were the same as those described in the foregoing section.

Preparation of Adsorbed Virus.—The brains of three mice which succumbed to experimental encephalomyelitis were ground in M/50 phosphate buffer, pH 6.6, to a 10 per cent suspension. This was centrifuged at 1500 R.P.M. for 25 minutes. To 2 cc. of the supernatant fluid were added 98 cc. of the Gel C (about 2 mg. Al_2O_3 per cc. of buffer). The mixture, which then consisted of active tissue in a dilution, by weight, of 1:500, was vigorously shaken and kept in the cold for 24 hours. It was next centrifuged and the deposit washed three times with 150 cc. of the buffer each time. Emphasis was laid on thorough washing, since insufficient cleansing might not have removed the excess, or unadsorbed, virus. The precipitate was resuspended in 200 cc. of Tyrode solution, pH 7; thereupon the suspension contained Al_2O_3 in a concentration of 1 mg. per cc.

When tissue cultures were used as antigens, 16.5 cc. of each cultured strain, after centrifuging to remove most of the cells, were added to 8.25 cc. of the gel (7.5 mg. per cc. of buffer). The mixture was thoroughly shaken and kept cold overnight. The alumina was sedimented by centrifugation and the deposit was washed thrice in 100 cc. of the buffer. It was then suspended in 80 cc. of Tyrode's solution pH 7; thus the concentration of Al_2O_3 remained approximately 0.75 mg. per cc.

When both final products were titrated by means of the mouse intracerebral tests, they were found to contain 3×10^3 to 3×10^4 m.i.u. of virus.

Western or Eastern strains, or both combined, differed in no way in antigenic power.

Inactivation of Virus.—A significant point is that the vaccines were not disease-producing as they were ordinarily employed; yet, when they were inoculated in the brain of mice and guinea pigs, or in the subcutis of guinea pigs in larger doses,

characteristic lethal encephalitis ensued. Moreover, active virus was recovered from the cutaneous nodules produced by injection of alumina material. Clearly then, the adsorption process as carried out did not inactivate the infective agent.

The Local Reaction.—The local reaction that developed at the site of injection was referable to the presence of the gel, for no distinction could be made between this reaction and the one induced by aluminum hydroxide alone. It is not improbable that the reaction is characteristic of the general group of insoluble chemical agents used as adsorbents. The alumina as such persisted beneath the skin for at least 3 weeks, during which time there was marked phagocytosis by polynuclear and monocytic cells. Later the involved area showed formation of granulation tissue including giant cells, together with invasion by lesser numbers of the leucocytes. Gradual retrogression in size occurred thereafter until the nodule disappeared in the 5th or 6th week. In instances in which 7.5 mg. or more of Al_2O_3 were used, a late sterile abscess supervened followed by ulceration, discharge of contents, and finally, healing by "secondary intention."

Although the gel virus in the immunizing dose did not produce encephalitis after subcutaneous inoculation, the infective agent was recovered from nodules excised on the 2nd and 4th but not on the 7th day after injection or later. The method employed for recovering the active agent from the lesions consisted in grinding the excised nodules to a fine pulp in a minimal amount of Tyrode solution and injecting it into the brains of six mice for each sample. No attempts at elution of the virus from the gel were made.

Effect of Storage.—The adsorbed material still retained its immunizing capacity after being stored in the cold for 2½ and 6 months—the longest periods studied—at which time it still contained virus. This was manifested by the lethal encephalitis induced in all of five mice after its intracerebral inoculation. How much virus was present could not be told since it was not found possible to elute the infective agent from the gel. In a sample which was stored for 5 weeks, however, a content of 3×10^4 m.i.u. was noted.

Immunization with Adsorbed Virus.—Proceeding as with the chemically untreated mouse virus, we attempted to determine the minimal immunizing dose of the adsorbed material.

Since it was difficult to elute the virus and titrate its concentration precisely, we undertook an experiment in which known quantities were added to the adsorbent, Gel C, and then used for immunization (Table VI).

One may infer that 3×10^4 m.i.u. of adsorbed virus in repeated doses are as adequate for immunization as like amounts of unadsorbed, mouse passage virus.

Immunization against Nasal, Subcutaneous, and Intracerebral Tests.—When a dose of 3×10^5 to 3×10^4 m.i.u. was injected subcutaneously, it was found to im-

munize guinea pigs to experimental nasal or subcutaneous infection, as shown in Table VII. For protection against the intracerebral test, three such injections were required, just as was found to be the case when unadsorbed virus was used. On the other hand, if 3 cc. of adsorbed material were given in one inoculum, instead of 1 cc. in three doses, then febrile reactions arose, accompanied by the presence of virus in the blood. For example, in one series of six guinea pigs receiving a single large inoculum, two animals died of virus infection and three of the four survivors succumbed to the usual intracerebral test (see also Table IV). This finding is in

TABLE VI

The Immunizing Capacity of Different Amounts of Virus Adsorbed on Alumina

No. of weekly subcutaneous injections	M.i.u. in each dose	Test for resistance* given intracerebrally about 1 month later	Result†
		M.L.D.	
2	$<3 \times 10^1$	$>10^4$	3/3
3	$<3 \times 10^1$	$>10^4$	3/3
2	$>3 \times 10^1$	$>10^4$	3/3
3	$>3 \times 10^1$	$>10^4$	3/3
2	$>3 \times 10^2$	$>10^4$	3/3
3	$>3 \times 10^2$	$>10^4$	2/3
2	$>3 \times 10^3$	$>10^4$	3/3
3	$>3 \times 10^3$	$>10^4$	1/3
2‡	$>3 \times 10^4$	$>10^4$	0/3
3	$>3 \times 10^4$	$>10^4$	0/3

* In the test for acquired resistance, the guinea pig brain passage virus which was employed induced lethal encephalitis in all of six guinea pigs inoculated intracerebrally with 1:50 to 1:500,000 dilutions and in all of nine mice receiving dilutions only to 1:5,000.

† Fractions defined in Table I.

‡ For the comparative observations on the capacity of one, two, and three injections to produce resistance to an intracerebral test inoculation, see similar experiments cited in Table VII.

agreement with what occurs when a similar injection of unadsorbed virus is given. It is plain that safer and more effective protection follows the use of repeated, small quantities of virus.

Febrile Reactions and Circulating Virus.—Eleven of forty-six animals receiving 3×10^4 m.i.u. of virus responded with fever, and of the eleven only two had blood infection. The incidence of febrile reactions and of circulating virus became greater with larger doses just as with unadsorbed virus.

An analysis of the experiments with alumina adsorbed virus leads to the conclusion that there is little, if any, difference in the effects

of the mouse passage, unadsorbed virus and of the Gel C adsorbed material respectively. A lesser rate of absorption *in vivo* of the adsorbed virus could not be discerned. Indeed, it was shown that the adsorption process as here employed does not inactivate the infective agent and that an adequate amount of the alumina material, that is, adequate in antigenic capacity yet within the range of least constitutional disturbances of fever and of circulating virus, is the same as with living mouse passage virus,—3×10^3 to 3×10^4 m.i.u. given three times at weekly intervals.

TABLE VII

Nasal and Subcutaneous Tests for Resistance, as Compared with Intracerebral

No. of weekly subcutaneous injections of absorbed virus	M.i.u. in each dose	Test for active immunity			
		M.L.D.	Given after last immunizing dose	By way of	Result*
			days		
1	3×10^3 to 3×10^4	1–5	23	Nose	1/10
1	3×10^3 to 3×10^4	1–5	23	Subcutis	0/10
1	3×10^3 to 3×10^4	10^3–10^4	12–30	Brain	9/12
2	3×10^3 to 3×10^4	10^3–10^4	12–30	"	3/6
3	3×10^3 to 3×10^4	10^3–10^4	12 days to 6 mos.	"	0/90

* Fractions defined in Table I. All of fifty normal control guinea pigs succumbed to the test inoculations.

Precipitated Virus as Immunizing Preparation

The type of precipitant chosen for this study was tannin (tannic acid).

Mode of Preparation.—The fresh brains obtained from six mice which died of the experimental disease were ground to an even suspension in 90 cc. of sterile distilled water and spun in an angle centrifuge at 3,000 R.P.M. for 20 minutes. To the supernatant fluid was added a sufficient amount of 2 per cent tannin to yield a final concentration of 0.2 per cent. The precipitate formed was thoroughly agitated and the material placed in the cold for 18 to 24 hours. It was then centrifuged and the deposit washed in 100 cc. of sterile saline solution, after which the precipitate was resuspended in 90 cc. of hormone broth, pH 7.6, and stored in the cold until used.

The bacteria-free suspension in the usual dosages of 1 cc. gave rise to no nodules or other visible local reaction in the skin of guinea pigs, nor was the experimental disease induced in any of more than fifty animals employed for these tests. Infec-

tion arose, however, in guinea pigs and in mice after the tannin precipitated virus was introduced directly into the brain. Precipitation by tannin, like adsorption by alumina, does not, therefore, inactivate the virus.

Titration by means of guinea pig or mouse intracerebral test, of freshly prepared precipitate revealed as a rule the presence of virus in dilutions up to 10^{-4}, and often higher. Active virus was recovered from specimens stored in the cold as follows: after 2 months, 30,000 m.i.u., after 3 months, 3,000 m.i.u., and after $3\frac{1}{2}$ months, approximately 300 m.i.u.

In the following experiments, the procedure in general followed that hitherto employed.

Immunization with Precipitated Virus.—A summary of the experimental results follows:

When less than 3×10^3 to 3×10^4 m.i.u. of such virus were injected subcutaneously, it was found that guinea pigs were not protected against experimental intracerebral infection with 10^3 to 10^4 M.L.D. A single injection of this dose sufficed, however, to protect nine of ten guinea pigs against 1 to 5 M.L.D. given intranasally 2 to 4 weeks later, and all of five animals against these doses which are ordinarily lethal when injected subcutaneously. So also, fifteen other guinea pigs acquired immunity to nose or skin infection by means of two and three immunizing doses injected at weekly intervals. For production of complete resistance to the introduction of 10^3 to 10^4 M.L.D. into the brain, a rigorous test, three such inocula at 7 day intervals were found necessary. All of twenty animals were thus protected, as shown by their resistance to experimental infection on the 10th to the 90th day after the last immunizing dose, although of fifteen untreated animals, all succumbed to the test inoculation.

This immunizing capacity of the tannin preparation was maintained during its storage in the cold for at least 2 months, at which time the material contained at least 3×10^4 m.i.u. per cc.

During the period of immunization, nine of thirty-eight guinea pigs receiving this quantity of precipitated virus exhibited the familiar, transitory fever on the 2nd to 4th day after inoculation, but in none was this accompanied by other signs of disease. In one of twenty-five treated animals virus was found in the blood by the described method.

Active tissue precipitated by tannin serves as an immunizing agent just as does adsorbed and unadsorbed mouse material. No evidence was obtained to indicate that the infective agent in the tannin preparation was inactivated by the process of precipitation, nor any that the rate of absorption of precipitated virus, subcutaneously injected, differed from that of adsorbed or chemically untreated virus.

SUMMARY AND CONCLUSIONS

Active Eastern or Western equine encephalomyelitis virus in three forms,—chemically untreated but simply passaged through series of mice; adsorbed on alumina Gel C, and precipitated by tannin,—yielded practically the same results when employed for the immunization of guinea pigs.

The virus is not inactivated by the process of adsorption or precipitation: guinea pigs and mice inoculated in the brain with these materials develop lethal encephalomyelitis in the same manner as when chemically untreated mouse passage virus has been used. Moreover, there is no difference in the rate of absorption *in vivo* of the chemically treated and untreated virus preparations. After storage of the three immunizing preparations—the longest periods thus far studied being 2 to 3 months for mouse passage and for precipitated suspensions, and 6 months for adsorbed material—each was found to contain an amount of virus sufficient to produce immunity in animals against the usual intracerebral test inoculation. Finally, the protection afforded by the three preparations is apparently durable, as is true of many active viruses utilized in preventive treatments.

The amount of the virus necessary to confer protection may be defined as that which immunizes (*a*) with the least number of antigenic units and (*b*) with the minimum of febrile reaction and blood infection. In proportion as this amount is exceeded, the incidence of fever and of circulating virus increases and, on the other hand, as this amount is decreased, the degree of induced immunity is diminished.

We have thus shown that for this particular virus and in the guinea pig, one or two subcutaneous doses of 1 cc. of any of the different virus preparations, each containing 3×10^3 to 3×10^4 mouse infective units, bring about protection regularly against experimental infection by way of the nose or subcutis. The results are irregular when the test is made by way of the brain. By three injections, resistance is invariably obtained against as many as 10^3 to 10^4 lethal doses, given intracerebrally.

No matter in what form the virus is given, as mouse passage, or adsorbed, or precipitated material, in certain instances fever occurs and virus circulates. With the amount of virus adequate for immuni-

zation (3,000 to 30,000 m.i.u.) a mild or subclinical infection may occur in the guinea pig without other manifestation of disease. Lesser quantities of virus apparently fail to gain a foothold in the animal and thus fail to bring about resistance.

To conclude, a quantitative basis has been established for the comparison of the immunizing capacities of preparations employed in experimental equine encephalomyelitis in guinea pigs.

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ON CROSS REACTIONS OF IMMUNE SERA TO AZOPROTEINS

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The overlapping reactions commonly observed in serological tests are ascribed by most authors to multiple antibodies formed as a result of the presence of several substances or distinct specific groups in the immunizing antigen. Yet studies on azoprotein immune sera have shown that antibodies corresponding to a particular compound regularly react with other substances which are sufficiently related in chemical structure (1, 2). Thus cross reactions in general can be understood on this principle alone without postulating a multiplicity of antibodies. However, this explanation does not cover the observations made when immune sera are partially exhausted by heterologous antigens.

It is unnecessary to cite examples of the well known and widely used fact that immune sera for bacteria and blood cells after treatment with a heterologous antigen (sensitive to its action) still react with the homologous antigen but no longer with that used for exhaustion. This method does not succeed as readily and regularly with precipitin sera for proteins (3). Yet in a number of experiments such sera could be made highly specific by fractional precipitation with heterologous antigen which reveals the presence of several antibodies. For instance, Hooker and Boyd (4) found that a precipitin for chicken serum acting also on duck serum, after removal of the precipitate formed on addition of the latter, still precipitated chicken serum intensely, but no longer duck serum. The assumption made here and in similar instances that the sera contain several antibodies directed toward different determinant groups in the protein has not been proved by chemical evidence. In order to investigate this question experiments were carried out with precipitins for artificially conjugated antigens

in which the chemical constitution of the reacting groups is known. For absorption, to avoid complications which may arise from the presence of an excess of heterologous antigen remaining in solution, insoluble antigens were chiefly used, namely blood stromata coupled with diazonium compounds (azostromata).

The essential result, *i.e.* the demonstration of the presence of multiple antibodies in an immune serum formed in response to an individual specific structure, has already been communicated briefly (2, 5). In another way, namely by the quantitative study of precipitin reactions, Heidelberger and Kendall (6) have arrived at similar conclusions.

Technique

Preparation of Antigens.—The azoproteins used for immunization were made as described previously (7, 8) using either horse serum or horse serum globulin as the protein component; about 6 millimols of the substance were coupled to 100 cc. of serum (or 7 gm. of protein) except in the case of para-aminosuberanilic acid in which twice the amount of substance was used. For purification the antigens were dissolved in water by means of dilute sodium hydroxide, and precipitated by an equal volume of alcohol and the requisite amount of acid. The precipitates were separated by centrifuging, washed several times with saline and were dissolved in saline with the aid of dilute sodium hydroxide, avoiding a large excess, and the solutions made neutral to litmus.

Tests.—The antigens used for the tests were prepared from chicken serum or casein as described previously (7, 8). The dilutions of the test antigens given in the tables are in terms of a 5 per cent stock solution. The intensity of the reactions is indicated as follows: 0, f. tr. (faint trace), tr. (trace), tr. (strong trace), ±, ±+, +, +±, ++, etc.

Coupling of Stromata with Diazonium Compounds.—For the preparation of stromata the method described by Sachs (9) was followed in the main. The blood corpuscles freed from serum proteins by repeated washings with saline were brought to twice the original blood volume with saline and kept at 56–60° for 40 minutes. Five volumes of distilled water were added and after thorough shaking and standing at room temperature for ½ hour enough 10 per cent salt solution was added to bring the salt concentration to 1 per cent. The stromata were separated from the liquid by centrifugalization and were washed repeatedly with saline until the liquid was free from hemoglobin. For coupling 0.1 millimol of the amino compound dissolved in 2 cc. of water and 0.3 cc. of N HCl was diazotized with 1 cc. N/10 sodium nitrite, and ⅔ of the solution was added to a suspension of 50 mg. of stromata in 5 cc. water and 0.6 cc. N sodium carbonate. After allowing the mixture to stand at 0–5° for ½ hour, the remainder of the diazotized substance and 0.3 cc. of N sodium carbonate were added and the coupling

allowed to proceed for another ½ hour (at 0–5°). After the addition of normal hydrochloric acid to weakly acid reaction to Congo red, the azostromata were centrifuged and washed several times with saline, the suspension made weakly alkaline to litmus by addition of normal sodium carbonate and the stromata washed with neutral saline until the supernatant liquids were perfectly colorless.

The azostromata used for absorption were prepared from rabbit blood, those for immunization from horse blood.

Immunization.—Rabbits were given daily intravenous injections of 2 cc. of the antigen solutions containing 10 mg. of azoprotein in 1 cc. Two or more courses of 6 daily injections were given at intervals of 1 week and the sera were tested 7 days after the last injection. It was found, furthermore, that satisfactory immune sera could be obtained by injecting suspensions of azostromata prepared by coupling the diazotized substances to stromata as described. Three to four courses of intravenous injections (2 cc.) were given as above of suspensions containing 2.5 mg. of azostromata in 1 cc. These sera gave precipitin reactions with the test antigens like sera produced with azoproteins; besides they were found to contain agglutinins and lysins for the blood from which the stromata were prepared. Absorptions with these blood corpuscles did not remove the precipitating antibodies.

Absorption Experiments.—Azostromata were packed by centrifuging and the sediment was mixed with concentrated or dilute immune serum by stirring. The mixture was allowed to remain at room temperature for 2 hours with occasional stirring, and the sediment was removed by centrifuging. Homologous and other specifically related immune sera produced distinct agglutination of the azostromata suspensions. The insolubility of the azostromata was demonstrated by testing the supernatant fluids of centrifuged suspensions and absorbed fluids with immune serum. In such tests no precipitation was observed, neither did the liquids give any yellow color on addition of alkali as is the case with even very dilute solutions of azoproteins.

Elution of Precipitins.—For example, 3 cc. of an immune serum (diluted 1:2 with saline) were absorbed with 2 mg. of azostromata. After centrifuging and washing twice with saline solution the stromata were suspended in 0.8 cc. of saline solution and 0.1 cc. of N/10 acetic acid was added. After 5 minutes the solution was freed from stromata by centrifuging and brought to pH 7.2 by addition of N/10 sodium hydroxide. The relative quantities of immune serum, azostromata and volume of eluent had to be determined for each serum.

Absorption Experiments

When an immune serum is treated with a properly chosen amount of one of several related antigens, giving reactions of different intensity, a fluid may be obtained which no longer reacts with the antigen used for exhaustion but still with those that have greater affinity for the immune serum, particularly with the homologous antigen. This

is illustrated in the tabulated experiments made with an azoprotein immune serum (Table I).

These effects can be explained by the diminution in antibody content without presuming that the immune serum contains more than one antibody. However, there are other observations in which such an

TABLE I

1.5 cc. of suberanilic acid (7) immune serum (No. 1) (diluted 1:4 with saline) were absorbed with 1 mg. of glutaranilic acid azostromata for 2 hours at room temperature; in the same way absorptions were made with adipanilic, pimelanilic and suberanilic acid azostromata. For the tests 0.2 cc. of the absorbed fluids was mixed with 0.04 cc. of the test antigen (dilution 1:125 of a 5 per cent solution).

Readings were taken after 1 hour at room temperature (first line) and after standing overnight in the ice box (second line).

Suberanilic acid immune serum absorbed with azostromata made from	Azoproteins made from casein and			
	p-Aminoglutaranilic acid B*-$(CH_2)_3$-COOH	*p*-Aminoadipanilic acid B-$(CH_2)_4$-COOH	*p*-Aminopimelanilic acid B-$(CH_2)_5$-COOH	*p*-Aminosuberanilic acid B-$(CH_2)_6$-COOH
p-Aminoglutaranilic acid	0	tr.	+	++
	0	tr.	+	++±
p-Aminoadipanilic acid	0	0	tr.	+±
	0	0	±	++
p-Aminopimelanilic acid	0	0	0	tr.
	0	0	0	±
p-Aminosuberanilic acid	0	0	0	0
	0	0	0	0
Unabsorbed immune serum	±	+±	++	++±
diluted 1:4 with saline	±	++	++±	+++

* B- represents $NH_2C_6H_4NHCO$.

explanation is excluded since the immune serum gave reactions of much greater specificity after partial absorption than on mere dilution. An example is given in Table II.

By cross absorption, using two heterologous azoproteins, the immune sera could be made specific for either antigen. Such experiments were performed, for example, with immune sera for *m*-aminobenzenesulfonic

TABLE II

Suberanilic acid immune serum (No. 2) (diluted 1:2 with saline) was absorbed with adipanilic acid azostromata as described in Table I. Similarly, a metanilic acid (1) immune serum (No. 1) was absorbed with *o*-aminobenzenesulfonic acid azostromata. The specificity of the absorbed fluids was compared with that of unabsorbed immune serum. From the immune serum diluted 1:2 with saline higher dilutions were made with saline and with normal rabbit serum (diluted 1:2), the object of using normal rabbit serum being to maintain a constant protein content.

Readings were taken after 1 hour at room temperature (first line) and after standing overnight in the ice box (second line).

Suberanilic acid immune serum	Azoproteins made from casein and		Metanilic acid immune serum	Azoproteins made from chicken serum and	
	p-Amino-adip-anilic acid	*p*-Amino-suber-anilic acid		*o*-Amino-benzene-sulfonic acid	Metanilic acid
Diluted 1:2 with saline, absorbed with adipanilic acid azostromata	0 0	++± +++	Diluted 1:2 with saline, absorbed with *o*-aminobenzenesulfonic acid azostromata	0 0	+± ++±
Unabsorbed, diluted 1:2 with saline	++ ++±	+++± ++++	Unabsorbed, diluted 1:2 with saline	++ ++	+++ +++
Unabsorbed, diluted 1:4 with saline	+ +±	++ ++±	Unabsorbed, diluted 1:4 with saline	± +±	+± ++±
Unabsorbed, diluted 1:3 with normal rabbit serum	+± ++±	++± +++	Unabsorbed, diluted 1:4 with normal rabbit serum	± +±	+± ++±
Unabsorbed, diluted 1:4 with normal rabbit serum	+ ++	++ ++±	Unabsorbed, diluted 1:5 with normal rabbit serum	tr. +	+ ++
Unabsorbed, diluted 1:6 with normal rabbit serum	± +	+ +±	Unabsorbed, diluted 1:6 with normal rabbit serum	tr. ±	+ +±

(metanilic) acid which were found to give cross reactions[1] with chemically related antigens as shown in Table III.

[1] Some of the immune sera gave definitely weaker cross reactions which were seen only after the test mixtures had been kept overnight in the ice box.

As will be seen from Table IV an immune serum for metanilic acid after absorption with *o*-aminobenzenesulfonic acid azostromata still precipitated *m*-aminophenylarsenic acid antigen. An analogous result was obtained when the experiment was set up in reversed order.

Equally striking was the result when more than two antigens were used (Table V). Here the supernatant fluids resulting from absorption with an heterologous azoprotein reacted on all other antigens while that used for absorption was not precipitated, or only very

TABLE III

2 drops of immune serum (No. 1) for *m*-aminobenzenesulfonic acid were added to 0.2 cc. of the antigens, diluted as indicated in the table.

Readings were taken after 1 hour at room temperature (first line) and after standing overnight in the ice box (second line).

Antigens made from chicken serum and	Position of the substituents						
	o-NH_2			*m*-NH_2	*p*-NH_2		
	Dilution			Dilution	Dilution		
	1:100	1:500	1:2500	1:500	1:100	1:500	1:2500
Aminobenzenesulfonic acid		+±		++±		tr.	
		+±		+++		±	
Aminophenylarsenic acid	0	0	0	+	0	0	0
	0	0	0	+	0	0	0
Aminobenzoic acid	0	0	0	±	0	0	0
	0	0	0	+	0	0	0

* R denotes the groups SO_3H, AsO_3H_2, COOH.

slightly; the homologous antigen, however, upon performing repeated absorptions removed the reactions with heterologous antigens first, finally the homologous one. The effects were not equally pronounced with individual metanilic immune sera but were always noticeable, depending, however, on the amount of absorbing material, since in general by repeated exhaustion with heterologous antigens all precipitin reactions were progressively diminished. With some antigens[2]

[2] Slight diminution of antibodies due to specific affinities is not easily demonstrable since treatment with any sort of azostromata will cause some reduction in antibody content.

TABLE IV

Metanilic acid immune serum (No. 1) was absorbed with *o*-aminobenzenesulfonic acid and with *m*-aminophenylarsenic acid azostromata using 2 mg. of stromata and 1.5 cc. of undiluted immune serum for each absorption. The tests were made as described in Table I.

Readings were taken after 1 hour at room temperature (first line) and after standing overnight in the ice box (second line).

Metanilic acid immune serum absorbed with azostromata made from	Azoproteins made from chicken serum and		
	o-Aminobenzenesulfonic acid	Metanilic acid	*m*-Aminophenylarsenic acid
o-Aminobenzenesulfonic acid	0 0	++ ++±	+ +±
m-Aminophenylarsenic acid	+± ++±	++± +++	0 0

TABLE V

Metanilic acid immune serum (No. 2) was absorbed with azostromata prepared from *o*-aminobenzenesulfonic, *m*-aminophenylarsenic and *m*-aminobenzoic acid azostromata, using 0.6 mg. azostromata for 1.5 cc. undiluted immune serum. The tests were made as described in Table I.

Readings were taken after 1 hour at room temperature (first line) and after standing overnight in the ice box (second line).

Metanilic acid immune serum absorbed with azostromata made from	Azoproteins made from chicken serum and			
	o-Aminobenzenesulfonic acid	Metanilic acid	*m*-Aminophenylarsenic acid	*m*-Aminobenzoic acid
o-Aminobenzenesulfonic acid	0 0	++± +++±	± ±	+ +
m-Aminophenylarsenic acid	+± ++	+++ ++++	0 0	+ +±
m-Aminobenzoic acid	+± ++	+++ ++++	± ±	0 f. tr.
Unabsorbed immune serum	++ +++	+++± ++++	+ ++	+± ++±

(*m*-arsanilic acid, *m*-aminobenzoic acid), reacting weakly, repeated absorption produced no significant diminution of the homologous reaction.

In the experiments presented the adsorption was carried out with

TABLE VI

Undiluted suberanilic immune serum (No. 3) was absorbed with adipanilic acid and sebacanilic acid azostromata (3 and 2 mg. respectively). The quantities used are indicated in the table. The tests were made as described in Table I.

Readings were taken after 1 hour at room temperature (first line) and after standing overnight in the ice box (second line).

1 cc. of suberanilic acid immune serum absorbed with azostromata made from	Azoproteins made from casein and		
	p-Aminoadipanilic acid	*p*-Aminosuberanilic acid	*p*-Aminosebacanilic acid
p-Aminoadipanilic acid	0 0	++± +++±	+± ++
p-Aminosebacanilic acid	+ +±	+++ ++++	0 0
Unabsorbed immune serum	+++ +++±	++++ ++++	+++ +++±

TABLE VII

Glycyl-leucine (GL) immune serum (No. 1) (diluted 1:3 with saline) was absorbed with glycyl-glycine (GG), *d*,*l*-leucyl-*d*,*l*-leucine (LL) and *d*,*l*-leucine (L) azostromata. In each case 1 mg. of azostromata was used for 1.5 cc. of diluted immune serum. The tests were made as described in Table I.

Readings were taken after 3 hours at room temperature (first line) and after standing overnight in the ice box (second line).

GL immune serum absorbed with azostromata made from	Azoproteins made from chicken serum and			
	GG	GL	LL	L
GG	0 0	+++ +++	+± ++±	++ ++±
LL	+ +	+++ +++	0 0	± +
L	± +	+++ +++	f. tr. tr.	0 0
Immune serum diluted 1:3, unabsorbed	+ +±	++++± ++++±	++ ++±	++± +++

blood stromata coupled with the respective diazonium compounds, but conformable results were obtained by partial precipitation with soluble azoproteins.

Similar observations were made with immune sera for suberanilic (7) acid and amino-benzoyl-glycyl-leucine (8) (Tables VI and VII). In some other cases, for example in the cross reactions of aminophenylarsenic antisera on antigens made from other phenylarsenic acids, no definite effects like those described above were obtained.

It should be mentioned incidentally that in azoprotein sera there are often, in addition to the antibodies specific for the azocomponent, others separable by absorption, which act on the original proteins (Heidelberger and Kendall (10)). The sera may also contain antibodies which precipitate azoantigens made from the same protein as the immunizing antigen and various diazonium compounds.

Elution of Absorbed Precipitins

After absorption of azoprotein immune sera with azostromata, as in the above experiments, precipitins can easily be liberated by treating the stromata with dilute acetic acid. These solutions, presumably, consist largely of antibody protein and have a low nitrogen content.

For instance, 6 cc. of metanilic acid immune serum were exhausted with 8 mg. metanilic acid azostromata; after several washings with saline, antibodies were liberated as described. The solution (6 cc.) which had half the potency of the original serum, titrated by diluting with normal rabbit serum, contained approximately 0.5 mg. protein per cc., indicating that the antibody protein in the serum amounted to about 0.7 per cent of the whole protein content, supposing that no significant non-specific adsorption of proteins had taken place.

In several cases the results obtained with antibodies separated by elution were in agreement with those already described, whereas in other instances (*e.g.* metanilic acid immune serum absorbed with *o*-aminobenzenesulfonic acid stromata) the purified antibody solutions obtained from a serum previously made specific by absorption with an heterologous stroma antigen again gave strong precipitation with the corresponding heterologous azoprotein, an apparent inconsistency. It was found, however, that the purified antibody solutions had more or less the tendency to precipitate non-specifically even azoproteins unrelated to the homologous antigen, probably owing to instability of the solutions. This difficulty could be overcome through increasing the protein concentration by the addition of normal rabbit serum; but since dilution with normal serum weakens the reactions, this method has so far not proved to be fully satisfactory for specificity tests.

Inhibition Tests

The specificity of the antibodies responsible for cross reactions of azoprotein sera was examined further by means of inhibition tests.

TABLE VIII

o-Aminobenzenesulfonic acid (1), metanilic acid (2), *m*-aminophenylarsenic acid (3) and *m*-aminobenzoic acid (4) were dissolved in water by the aid of sodium hydroxide and the solutions were made neutral to litmus and brought to the required volume. In test I, 0.1 cc. of solutions containing 1 millimol in 10 cc., in tests II, III and IV, 0.05 cc. of solutions containing $^1/_8$ millimol in 10 cc., was mixed with 0.2 cc. of the test antigens (prepared with chicken serum) diluted 1:500, and 0.1 cc. of metanilic acid immune serum (No. 2). The control tube contains only antigen and immune serum.

Readings were taken after 20 minutes (first line) and 1 hour (second line) at room temperature, and after standing overnight in the ice box (third line).

Test antigens made from....	I. Metanilic acid					II. *o*-Aminobenzene-sulfonic acid			III. *m*-Aminophenylarsenic acid			IV. *m*-Aminobenzoic acid		
Substances used for inhibition tests.....	1	2	3	4	Control	1	2	Control	2	3	Control	2	4	Control
	f. tr.	0	++	+±	++	f. tr.	f. tr.	+±	0	±	+	0	±	+
	±	tr.	++	+±	++±	tr.	f. tr.	++	0	±	+	0	±	+
	++	+±	+++±	+++	+++±	+±	±	++±	±	+±	++	+	+±	++

TABLE IX

Neutral solutions were prepared of the *p*-nitro derivatives of glutaranilic (1), adipanilic (2), pimelanilic (3), suberanilic (4) and sebacanilic acid (5), as described in Table VIII (C designates control). In tests II and VII, 0.05 cc. of the solutions containing $^1/_4$ millimol in 10 cc., in tests III, IV, V, VI and VIII, 0.05 cc. solutions containing $^1/_{16}$ millimol in 10 cc., and in test I, 0.05 cc. of solutions containing $^1/_{32}$ millimol in 10 cc. were mixed with 0.2 cc. of the test antigens (prepared with casein) diluted 1:500 and 2 drops of adipanilic acid or suberanilic acid immune serum.

Readings were taken after 15 minutes (first line) and 1½ hours (second line) at room temperature.

	Adipanilic acid immune serum and test antigens made from																			
	I. *p*-Aminoglutaranilic acid					II. *p*-Aminoadipanilic acid					III. *p*-Aminopimelanilic acid					IV. *p*-Aminosuberanilic acid				
Substances used for inhibition tests	1	2	3	4	C	1	2	3	4	C	1	2	3	4	C	1	2	3	4	C
	tr.	f. tr.	tr.	tr.	±	±	0	0	tr.	++	±	0	f. tr.	tr.	+	±	0	f. tr.	f. tr.	+
	tr.	f. tr.	tr.	±	+	+	0	tr.	±	++±	+	f. tr.	tr.	tr.	++	±	f. tr.	tr.	tr.	+±

	Suberanilic acid immune serum (No. 1) and test antigens made from																			
	V. *p*-Aminoadipanilic acid					VI. *p*-Aminopimelanilic acid					VII. *p*-Aminosuberanilic acid					VIII. *p*-Aminosebacanilic acid				
Substances used for inhibition tests	2	3	4	5	C	2	3	4	5	C	2	3	4	5	C	2	3	4	5	C
	f. tr.	0	0	0	+	+	tr.	0	tr.	+±	+	±	0	f. tr.	++	+	tr.	0	0	+±
	±	tr.	0	f. tr.	+±	+	±	tr.	±	++	++	+	0	tr.	++±	+±	±	0	tr.	++

TABLES X*a* AND X*b*

Neutral solutions were prepared of the *p*-nitrobenzoyl derivatives of glycine (G), *d,l*-leucine (L), glycyl-glycine (GG), glycyl-*d,l*-leucine (GL), *d,l*-leucyl-glycine (LG) and *d,l*-leucyl-*d,l*-leucine A (LL), as described in Table VIII (C designates control).

For the inhibition tests 0.05 cc. of the solutions (the concentration in millimols per 10 cc. is indicated in the tables) was mixed with 0.2 cc. of the test antigens (prepared from chicken serum) diluted 1:500 and two drops of immune serum.

TABLE X*a*

Immune sera for azo-proteins made from *p*-aminoben-zoyl peptides	Test antigens: Substances tested for inhibition	G				GG							LG			
		G	GG	LG	C	G	L	GG	GL	LG	LL	C	G	GG	LG	C
		Concentration 1:4				Concentration 1:4							Concentration 1:64			
G		0 0	+± +±	+± ++	++ ++±	0 0	+ +±	tr. tr.	+ +±	± ±	+ +±	+ +±	0 0	± ±	tr. ±	± +
		Concentration 1:16				Concentration 1:4							Concentration 1:4			
LG		tr. +	± +±	0 tr.	+± ++	+± ++	++ ++±	tr. +	++ ++±	0 tr.	++ ++±	++ ++±	++ ++±	++ ++±	0 tr.	++± +++

TABLE X*b*

Immune sera for azo-proteins made from *p*-amino-benzoyl peptides	Test antigens: Substances tested for inhibition	L					GG							GL					LL				
		L	GG	GL	LL	C	G	L	GG	GL	LG	LL	C	L	GG	GL	LL	C	L	GG	GL	LL	C
		Concentration 1:4												Concentration 1:16					Concentration 1:32				
L		0 0	++ ++±	+ +±	± +±	++ ++±								0 0	++ ++±	± +±	± +±	++ ++±	0 0	± ++	0 tr.	0 f. tr.	± ++
		Concentration 1:16					Concentration 1:64							Concentration 1:4					Concentration 1:64				
GL		f. tr. ±	+± ++	0 0	0 tr.	+± ++	+ +±	+ +±	± ±	0 0	+ +±	+ +±	+ +±	++ +++	++ +++	0 0	+± ++±	++ +++	+ +	+ +±	0 0	tr. +	+ +±

As is seen from Tables VIII, IX, X *a* and X *b* the reactions on heterologous antigens are regularly inhibited more strongly, or not less, by the simple substances corresponding to the specific part of the homologous antigen than by heterologous substances, *e.g.* the reaction of metanilic acid immune sera on *m*-arsanilic acid antigen was more inhibited by metanilic acid than by *m*-arsanilic acid, etc.

In inhibition tests made with sera which previously had been absorbed with an heterologous antigen it was found that the reactions of such absorbed sera with the homologous antigen were still inhibited by the simple substances corresponding to the heterologous antigen used for absorption. This shows that the remaining antibodies have affinity for the absorbing antigen, although no precipitation occurs.

DISCUSSION

From the experiments presented one may conclude that the increase in specificity after absorption of azoprotein immune sera with heterologous antigens is referable to a multiplicity of antibodies. Particularly striking is the fact—which cannot well be understood on the assumption of a single antibody—that by exhaustion with various heterologous antigens fluids of different specificities can be prepared. The objection that some of the absorbing antigen may pass into solution and interfere with the reaction seems to be ruled out by the insolubility of the antigens made from stromata; of course, an alteration of antibodies by mere contact with the insoluble material would seem highly improbable.

As already mentioned, when results similar to those described are obtained with natural antigens, it is usually concluded that the antibodies present in one immune serum are severally directed toward distinct groupings or components of the antigen. This view while possible in cases of natural antigens is not tenable in those under discussion. For example, the cross reactions of metanilic acid immune sera are evidently due either to the presence of SO_3H or to acid groups in meta position (see Table III). But the antisera are not strictly specific for the SO_3H radical itself since they do not react with all aromatic sulfonic acids; likewise meta position of substituents, regardless of their nature, is not sufficient to cause a positive reaction. Thus it appears that the specificity is determined by the structure of the metanilic acid

molecule as a whole. Clearly, there are not several specific groupings in the immunizing antigen, identical with groupings contained in the positively reacting heterologous antigens, to which the antibodies could be specifically related. The same consideration holds for the reactions of suberanilic acid immune sera on sebacanilic acid antigen.

The view just outlined is supported by the inhibition tests described. The reactions show that, although the immune sera are made up of fractions somewhat different in their specificity, *i.e.* in the affinity to various heterologous antigens, the antibodies all have maximal affinity for the immunizing antigens, the cross reactions, as a rule, being inhibited best by the homologous simple substances.

It can be concluded therefore, that the separation from an immune serum of antibody fractions, different in specificity, is no proof of the existence of several substances or special groups in the immunizing antigen. In other words, antibodies formed in response to one antigen, although adjusted to a certain structure, are not entirely uniform but vary in specificity to some degree. An additional argument in favor of this interpretation is the fact established repeatedly, that immune sera produced in animals of the same species may differ in their reactions with heterologous antigens. In this connection reference may be made to the presence in one serum of antibodies varying in their avidity as shown by the influence of temperature on the reactions.

The results under discussion have some bearing on absorption experiments with natural antigens. Thus one must expect that a separation of antibody fractions will be possible even when an inciting natural antigen does not contain several determinant structures; this might explain why such effects are to some extent obtained almost regularly. Evidence to show that the demonstration of divers antibody fractions is no decisive proof of the presence of several separable substances in the corresponding antigen has been brought forward by one of the authors and Furth (11), and by Burnet (12).

If the complexity of an antigen cannot be established chemically in individual cases it will not always be easy to determine the cause for the production of multiple antibodies, following the injection of an antigenic material. The assumption of several substances or determinant groups will be less probable if on continued exhaustion with

an heterologous substance the homologous reaction tends to disappear, as was mostly the case in the experiments here reported.

SUMMARY

The phenomena observed on absorption of immune sera to azoproteins with heterologous azoantigens lead to the conclusion that in general the sera do not contain a single antibody but antibody fractions somewhat different in their reactivity for heterologous antigens. From the constitution of the azocomponent it follows that these fractions cannot be specific for distinct chemical groups in the molecule. In fact, inhibition experiments showed that in the cases examined the various antibodies are all specifically directed towards the whole molecule of the homologous hapten.

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STUDIES ON CULTURE STRAINS OF EUROPEAN AND MURINE TYPHUS

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The cultivation of the rickettsiae of European and murine typhus reported in a previous paper (1) has provided a new method whereby the pathogenicity of these two strains can be compared. Previous studies on the pathogenicity of various strains have entailed a comparison of the results obtained by injecting brain emulsions and testicular washings. The great difference in the number of rickettsiae in the suspensions from the tunica vaginalis and brain makes such a comparison not altogether satisfactory. It was thought that more reliable results could be obtained by studying both strains in cultures prepared in the same manner. The possibility of the European typhus virus undergoing a transformation toward the murine type as the result of prolonged cultivation in a medium containing tunica tissue also seemed worth investigating during the course of this work (Mooser *et al.* (2)).

Technique

Initiation of Cultures of European Typhus Rickettsiae.—Guinea pig 1-16, which had been inoculated with European typhus (Breinl strain[1]) was sacrificed 6 days after injection, just as the febrile reaction had begun, the temperature being 39.7°C. The testicles, aseptically excised, showed a slight amount of thin exudate and a few petechiae. Stained preparations of this exudate revealed a few typical intracellular rickettsiae. The tunica was stripped from the testicles, minced with scissors, then ground with a small amount of sterile saline. The turbid liquid thus obtained was used to inoculate normal minced tunica for the initial cultures of this strain.

Initiation of Cultures of Murine Typhus Rickettsiae.—This has been described in a previous report (1).

Cultures.—The tunica-serum-Tyrode medium was used in these cultures which were prepared, stoppered, and incubated at 37°C. as previously described (1, 3).

[1] This strain was very kindly furnished by the United States Public Health Service at Washington, D. C.

Tests for Virulence.—The inoculation of guinea pigs with culture material has likewise been described in the previous papers.

EXPERIMENTAL

In order to determine the virulence and pathogenicity of the cultures, a portion of a single flask of culture material was injected intraperitoneally into guinea pigs. The European strain has been carried in cultures for 1½ years through 36 generations, while the murine strain has been carried for over 4 years through 82 generations. Giemsa stained preparations from the first generation cultures of the European strain, after 1 week's incubation, showed as many rickettsiae as initial cultures of murine strains. Beginning with the second culture generation, when the rickettsiae became very numerous, they have not diminished in either strain. Both strains were transferred at intervals varying from 1 to 10 weeks, the cultures being kept either at 37°C. or in a freezing box in which the temperature was maintained at −10° to −20°C. (3).

It will be noted from Table I that the febrile reaction and incubation periods following the intraperitoneal injection of culture material from each strain showed very little variation from the first few to the later generations in culture, whence it may be concluded that the virulence of the cultures remained quite constant throughout the period of cultivation represented in Table I, *i.e.*, 1 year for the European and 4 years for the murine strain.

The pathogenicity of the murine strain has remained remarkably constant throughout the 4 years of cultivation. In the case of the European strain, the scrotal lesions produced by culture material were distinctly more severe than those observed in guinea pigs inoculated with brain emulsions of the corresponding passage strain (*cf.* Table III), probably because the number of rickettsiae present in the culture material injected is much greater than in the customary inoculum of brain emulsions as used for carrying European typhus in guinea pigs. However, it will be noted that after the 19th generation in culture, the scrotal lesions seemed to become somewhat less intense, although the febrile reactions were fully as severe as in the earlier cultures. Whether this signifies a real change in pathogenicity of the rickettsiae as the result of prolonged cultivation *in vitro* will require further investigation.

Since the dose of injected rickettsiae was great, producing marked scrotal lesions, it was thought that a more definite evaluation of the pathogenicity of the rickettsiae after cultivation *in vitro* could be

TABLE I

Virulence and Pathogenicity of Successive Generations of Cultures of European and Murine Typhus

Guinea pigs inoculated with culture material						Guinea pigs inoculated with emulsions of brain from others infected with culture material				
No.	Generation of culture	Maximum temperature	Interval between injection and maximum temperature	Scrotal reaction	Interval between maximum temperature and transfer	No.	Amount of brain injected*	Maximum temperature	Interval between injection and maximum temperature	Scrotal reaction
				Cultures of European Typhus						
		°C.	days		days			°C.	days	
1-31	1	40	7	Marked						
8-44	1	40.2	5	Moderate						
7-48	3	40.5	5	"						
1-36	3	39.9	5	Marked						
1-60	6	40.6	8	Moderate to marked	5	1-71	1/10	40	8	None
1-59	6	40.7	3	Marked						
2-11	8	40.5	3	"						
2-12	8	41	3	Moderate	5	2-19	1/10	40.5	10	Slight to moderate
						2-20	1/5	41	12	None
2-39	12	39.8	6	"						
2-40	12	40.2	6	None						
2-47	13	40.6	3	Moderate to marked	4	2-58	1/5	40.5	6	"
2-48	13	40.4	3	Marked		2-59	1/10	40.4	10	Moderate
2-78	17	40.3	5	"	None	2-82	1/5	40.3	8	Slight
						2-83	1/10	40.6	6	None
2-88	19	40.3	3	Moderate to marked	2	2-92	1/5	41	9	Slight to moderate
						2-93	1/10	40	9	None
2-89	19	40.8	2	Marked	3	2-94	1/5	40.5	8	"
3-02	21	40.2	4	Moderate		2-95	1/10	40.4	7	"
3-03	21	40	6	Slight						
3-07	22	40	2	None						
3-06	22	40.2	4	Moderate						
3-13	23	40	3	"	3	3-18	1/10	40.2	9	"
						3-19	1/5	40.4	9	Slight to moderate

* The figures in this column refer to the fraction of the entire brain, injected as a saline emulsion.

TABLE I—*Concluded*

Guinea pigs inoculated with culture material						Guinea pigs inoculated with emulsions of brain from others infected with culture material				
No.	Generation of culture	Maximum temperature	Interval between injection and maximum temperature	Scrotal reaction	Interval between maximum temperature and transfer	No.	Amount of brain injected*	Maximum temperature	Interval between injection and maximum temperature	Scrotal reaction
Cultures of European Typhus—*Continued*										
		°C.	*days*		*days*			°C.	*days*	
3-12	23	40	4	None						
3-26	25	40.6	4	Moderate						
						3-30	1/5	40.2	7	None
3-27	25	41	6	"	1	3-31	1/5	40.4	8	Slight
						3-32	1/10	40.5	11	"
3-39	27	40	2	Slight to moderate	None	3-45	1/10	40.7	6	None
						3-46	1/5	40.2	12	"
3-40	27	40.2	2	Moderate						
3-59	29	40.3	7	"	1	3-62	1/5	40	7	Moderate
						3-63	1/10	38.8		None
Cultures of Murine Typhus										
40-50	1	40.5	6	Marked						
47-12	1	40	6	Moderate						
47-20	1	40.6	4	Marked						
25-35	2	40.1	6	"						
33-65	5	40.5	7	"						
33-62	5	40.8	7	Moderate						
34-70	11	40.4	6	Marked						
35-13	15	40.4	6	"						
35-68	20	41.1	3	"						
36-80	27	41	3	"						
38-62	32	40.3	7	"						
39-31	34	40.7	5	"						
43-79	52	40.5	3	"						
						46-42	1/5	40.2	5	Marked
47-04	60	40.5	5	"		46-75	1/5	40.7	5	Moderate
						51-92	1/10	39.8	9	Moderate to marked
51-88	70	40.8	3	"		51-93	1/5	40.5	8	Marked

obtained by transferring the infection produced by the injection of culture material to other guinea pigs, by injecting a measured amount of brain tissue. In Table I, it will be noted that when the culture virus was carried through the first guinea pig into the second (by means of brain emulsions) the febrile reactions in the second series of both strains were on the whole as pronounced or greater than in the first series which had been injected with culture material. This would seem to indicate that virulence was maintained for at least two generations of guinea pigs although the European strain showed an apparent diminution in the intensity of the scrotal reactions in the second generation.

The question of altered pathogenicity consequent to prolonged cultivation *in vitro* was more extensively studied in experiments summarized in Table II. It will be seen that the culture material from the 23rd generation of the European strain produced in guinea pig 3-13 a typical febrile reaction with moderate scrotal involvement. The brain of this animal, injected into guinea pigs 3-18 and 3-19 produced slightly higher temperatures, but the scrotal reaction was less intense in one animal and entirely absent in the other. Brain transfers into the 3rd generation animals (Nos. 3-22 and 3-23) and likewise into the 4th (Nos. 3-24 and 3-25) resulted in febrile reactions and scrotal lesions about like those in the animal (No. 3-13) infected with the culture material. Similar results were obtained with the 25th generation cultures carried through six guinea pig generations, with the 27th generation cultures carried through four guinea pig generations, and with the 29th generation cultures carried through five guinea pig generations. In general, the intensity of the scrotal lesions, after the 19th generation in culture, did not vary significantly from that observed in the passage strain (*cf.* Table III).

A similar experiment with the murine strain was begun with the 27th culture generation, injected into guinea pig 36-80, and continued through five generations of guinea pigs. It will be noted from Table II that the amount of brain used for transferring the infection in this experiment was gradually diminished from 1/5 to 1/160 part of the whole brain. In spite of the decreased dosage, the febrile reactions, incubation periods, and the scrotal lesions remained, on the whole, unaltered. This experiment would seem to show that the murine

TABLE II

Virulence and Pathogenicity of Cultures of European and Murine Typhus in Successive Generations of Guinea Pigs

Generation of culture	Guinea pig No.	Amount of brain injected	Maximum temperature	Interval between injection and maximum temperature	Scrotal reaction	Interval between maximum temperature and transfer
			European Typhus			
			°C.	*days*		*days*
23	3-13		40	3	Moderate	3
	↓					
	3-19	1/5	40.4	9	Slight to moderate	
	3-18	1/10	40.2	9	None	None
	↓					
	3-22	1/10	40	7	Slight	
	3-23	1/5	39.8	6	Slight to moderate	None
	↓					
	3-24	1/5	40.3	8	None	
	3-25	1/10	40.4	8	Moderate	
25	3-27		41	6	"	1
	↓					
	3-30	1/5	40.2	7	None	
	3-32	1/10	40.5	11	Slight	
	3-31*	1/5	40.4	8	None	2
	↓					
	3-37	1/5	40.2	5	Moderate	
	3-38	1/10	40.8	7	"	None
	↓					
	3-44	1/5	40	7	Slight	
	3-43	1/10	40.2	8	None	None
	↓					
	3-47	1/5	40.2	9	Slight	
	3-48	1/10	40.5	9	Moderate	None
	↓					
	3-53	1/10	40.5	7	Slight	
	3-54	1/5	40.6	7	Slight to moderate	
27	3-39		40	2	" " "	2
	↓					
	3-46	1/5	40.2	12	None	
	3-45	1/10	40.7	6	"	3
	↓					
	3-52	1/5	40.2	9	Slight to moderate	
	3-51	1/10	40.6	9	None	None
	↓					
	3-57	1/10	40	7	Slight	
	3-58	1/5	40.6	8	Moderate	

* Brain of guinea pig 3-31 was frozen for 4 days before injecting into guinea pigs 3-37 and 3-38.

↓ Indicates transfer of infection from the guinea pig above to those directly below this symbol.

TABLE II—*Concluded*

Generation of culture	Guinea pig No.	Amount of brain injected	Maximum temperature	Interval between injection and maximum temperature	Scrotal reaction	Interval between maximum temperature and transfer
			°C.	*days*		*days*
European typhus—*Continued*						
29	3-59		40.3	7	Moderate	1
	↓					
	3-63	1/10	38.8		None	
	3-62	1/5	40	7	Moderate	2
	↓					
	3-66	1/10	40	7	None	
	3-67	1/5	40.5	9	"	2
	↓					
	3-71	1/10	40	9	"	
	3-70	1/5	40.2	7	"	3
	↓					
	3-74	1/10	39.8	15	"	
	3-75	1/5	40.5	7	Marked	
Murine Typhus						
27	36-80		40	3	Marked	2
	↓					
	36-81	1/5	39.9	8	"	
	36-82	1/10	40.4	11	"	None
	↓					
	36-94	1/10	39.8	10	None	
	36-95	1/20	40.8	10	Marked	2
	↓					
	37-56	1/10	40.4	9	"	
	37-57	1/20	40.5	9	"	None
	↓					
	37-64	1/20	40.4	11	"	
	37-65	1/20	40	11	"	None
	↓					
	37-77	1/80	40.4	11	"	
	37-78	1/160	40 5	11	Moderate	

typhus is characterized by a definite predilection for the tunica tissue in guinea pigs, since much larger doses of brain (*i.e.* rickettsiae) in European typhus almost never show such intense scrotal lesions. Since the brain lesions are distinctly less numerous (judging from histo-

logical sections) in murine typhus in guinea pigs than in European typhus (4, 5), it has been quite generally held that a measured amount of brain emulsion of the former contains correspondingly fewer rickettsiae, so that injection of brain from guinea pigs infected with murine typhus might result therefore in milder infections than the

TABLE III

Successive Brain Transfers of the Passage Strains of European and Murine Typhus

Guinea pig No.	Amount of brain injected	Maximum temperature	Scrotal reaction	Interval between maximum temperature and transfer
		European Typhus		
		°C.		*days*
2-35	1/10	40.2	None	3
↓				
2-46	1/5	40	"	
2-45	1/10	39.8	Slight to moderate	None
↓				
2-54	1/5	40.6	None	
2-53	1/10	40.2	Slight to moderate	None
↓				
2-65	1/5	39.8	Slight	
2-64	1/10	39.8	None	None
↓				
2-70	1/10	40.2	Moderate	
2-71	1/5	40.6	Slight to moderate	None
↓				
2-77	1/5	40	None	
2-76	1/10	40.2	Slight to moderate	1
↓				
2-81	1/15	38.6	None	
2-80	1/10	40.4	"	1
↓				
2-84	1/10	40	"	
2-85	1/5	40	"	None
↓				
2-86	1/5	40	Moderate	
2-87	1/10	40.2	Slight	None
↓				
2-91	1/5	40.2	None	
2-90	1/10	40.4	Slight to moderate	None
↓				
2-98	1/10	40.4	" " "	
2-99	1/5	40.4	None	

TABLE III—*Concluded*

Guinea pig No.	Amount of brain injected	Maximum temperature	Scrotal reaction	Interval between maximum temperature and transfer
		Murine Typhus		
		°C.		*days*
51-96		40.4	Marked	
↓				
54-38	1/10	40.2	Slight	
53-33	1/5	41.5	Marked	1
↓				
55-01	1/10	40.2	Slight to moderate	
55-02	1/5	40.2	Marked	2
↓				
55-07	1/10	40.2	"	
53-44	1/5	40.6	"	None
↓				
54-69	1/5	40.4	"	
55-08	1/10	40.5	"	1
↓				
54-64	1/5	39.6	None	
55-09	1/10	40.3	Moderate	3
↓				
55-11	1/10	40.2	Marked	
54-98	1/5	40.2	"	1
↓				
54-97	1/5	40.2	Slight	
55-13	1/10	40.2	Moderate	1
↓				
56-25	1/5	40.4	Slight to moderate	
55-15	1/10	40.4	Moderate	1
↓				
55-18	1/5	40.5	"	
56-31	1/10	40	Moderate to marked	2
↓				
55-23	1/5	40	Slight	
55-22	1/10	40	Marked	

same dose of European typhus brain. This, however, as seen from the foregoing, was not the case.

To determine whether the intensity of the scrotal lesions observed for the murine typhus as summarized in Table II was possibly due to an enhancement of the virulence consequent upon prolonged cultivation in a medium containing tunica tissue, a comparison was

made of the passage strains of murine and European typhus. Each strain was carried through 10 successive generations of guinea pigs, using the same dosage of brain emulsions for the inoculations in both series. The results are summarized in Table III. In the European typhus series, scrotal lesions were entirely absent in about half of the animals and in the remainder distinctly less severe than in murine typhus. In the murine series, guinea pig 55-22, representing the 10th successive transfer by means of brain material, showed just as marked scrotal lesions as animals regularly injected with tunica material rich in rickettsiae. Indeed, a careful examination of the data in Table III would seem to indicate that the pathogenicity was rather enhanced in successive passages. For the first few transfers, 1/5 brain produced more severe infections quite regularly than 1/10 brain, whereas in the later generations more severe infections seemed to be produced by 1/10 brain. That a larger inoculum should induce a milder infection than a smaller one is perhaps explicable on the assumption that in the larger inoculum there may be a relatively higher antibody content. These experiments show that the scrotal lesions observed upon successive transfers of the murine culture strain through guinea pigs (*cf.* Table II) are typical of the passage strain and are not to be ascribed to enhancement of virulence during cultivation.

Thus it would seem from the experiments summarized in Tables I, II, and III that scrotal lesions are conditioned not only by the number of rickettsiae injected but also quite definitely by an inherent pathogenic property, characteristic of the murine strains.

SUMMARY

1. A strain of European typhus (Breinl) has been carried in cultures by means of successive transfers for a period of $1\frac{1}{2}$ years, the rickettsiae in such cultures being quite as numerous as in similar cultures of murine strains of typhus.

2. The virulence of the cultures of European typhus has remained constant throughout the period of cultivation, although, on the whole, the scrotal lesions caused by the later culture generations were somewhat less marked than those produced by the first generations.

3. A strain of murine typhus has been similarly carried in cultures for 4 years with no apparent loss in pathogenicity.

4. The characteristic scrotal lesion in murine typhus in guinea pigs is apparently referable to actual predilection of this strain for the tunica tissue rather than to the number of rickettsiae injected.

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STUDIES ON THE ETIOLOGY OF RABBIT POX

III. Tests of the Relation of Rabbit Pox Virus to Other Viruses by Crossed Inoculation and Exposure Experiments

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Plates 27 and 28

(Received for publication, September 26, 1935)

In the first paper of this series (1) the experimental transmission of rabbit pox from spontaneous cases of the disease and the pathogenic properties of the causative agent, a filterable virus, were reported. The clinical characteristics of the experimentally induced disease which were described in the second paper (2) were found to be indistinguishable from cases of spontaneous pox.

The work on experimental pox included experiments on the immunological aspects of the reaction to the infection, in which connection comparisons with other filterable viruses were made. The experiments which dealt with the inoculation of recovered (immune) rabbits are reported in this paper. In this study the viruses of pox, vaccinia, virus III disease, and infectious myxoma of rabbits were used in various combinations. The results on the exposure of recovered rabbits to clinical cases of pox are also presented, the original infections in these cases being pox, vaccinia, or virus III disease. And finally, the results on the cutaneous reaction of a calf to inoculation with pox, dermovaccine, culture (dermo) vaccine, and neurovaccine are also included. The following paper contains the results of serum-virus neutralization experiments (3).

Materials and Methods

Rabbit Pox Virus.—Tissue emulsions were prepared from the testicles of rabbits with an acute pox orchitis. The animals for the most part belonged to the

regular passage series of the Xy171 strain of virus and had been inoculated intratesticularly (1). 10 to 15 per cent emulsions by weight were made with Locke's solution by grinding the tissues with alundum. In the case of Berkefeld V tissue filtrates, bacterial sterility was tested against *B. prodigiosus* in the usual manner (1).

Vaccine Virus.—The vaccine virus used for the majority of the experiments was the New York City Board of Health virus grown in tissue culture for many generations by Dr. T. M. Rivers and then usually passed for 1 or more generations in rabbits by intratesticular injection. Neurovaccine virus which was carried in rabbits by intratesticular inoculation was also obtained from an early acute orchitis. With both specimens of vaccine virus, 10 to 15 per cent emulsions of testicular tissue were prepared in the same manner as pox virus; the tissue emulsions were not filtered.

In the experiments in which a calf was inoculated, fresh vaccine lymph supplied by the New York City Board of Health was used. It was labelled "670. Good until 5–6–33." It was used on Apr. 19, 1933.

Virus III.—The source of this virus was also an early acute orchitis following intratesticular inoculation. The tissue emulsions employed were prepared in the same manner as those of pox and vaccine viruses. The emulsions were not filtered.

Infectious Myxoma.—A 10 per cent unfiltered tissue emulsion of the virus was employed. It was injected intradermally in 0.25 cc. doses.

Animals.—All experiments with one exception were carried out on rabbits, most of which were hybrid stock purchased from dealers. The majority were male animals 4 to 6 months of age. For one experiment a 75 kilo Jersey Holstein calf 3½ months old was used.

Dosage and Route of Injection.—These features of inoculation are stated in connection with the description of the various experiments.

Immunity of Recovered Pox Rabbits to Reinoculation with Pox Virus

The reaction of recovered pox rabbits to reinoculation with pox virus was investigated in 27 cases; 13 animals had had the spontaneous and 14 the experimental infection.

In the spontaneous cases reinoculation was carried out 2 to 7 weeks after symptoms had been noted, and in the experimentally infected rabbits 49 to 97 days or a mean of 64 days after inoculation. The animals were distributed in 8 experiments; in 4, comprising 9 rabbits, Berkefeld V tissue-virus filtrates were used, and in 4 experiments on 18 rabbits, unfiltered tissue emulsions were employed. For the majority of rabbits, intradermal injections of 0.1 to 0.3 cc. were given; both full strength virus and dilutions ranging from 1:10 to 1:100,000 were employed and a total of 91 sites were injected. 1 animal was injected intravenously with 1.0 cc. and both corneas were scarified and 3 rabbits were injected intratesticularly with 0.5 or 1.0 cc. doses; full strength virus emulsion was used for these cases.

Each experiment included normal control animals injected by similar routes but usually with smaller doses than those employed for the recovered rabbits.

The results of all reinoculation tests were uniformly negative in contrast to the uniformly positive results in the normal controls. An example of these findings is given in Table I. In no instance was rein-

TABLE I

Results of the Intradermal Injection of Rabbit Pox Virus (Unfiltered) in Rabbits Recovered from Rabbit Pox

Rabbit	Virus dilutions 0.2 cc. dosage	Days after injection (Rectal temperature (°F.))						
		1	2	3	4	5	6	7°
		104.4°	102.9°	103.1°	102.7°	103.0°		101.2°
Recovered spontaneous case	1.0	±	++	+	+	±	0	0
	0.1	0	±	0	0	0	0	0
	0.01	0	±	0	0	0	0	0
		103.1°	101.5°	102.6°	102.7°	102.6°		102.7°
Recovered experimental case	1.0	+	++	++	+	0	0	0
	0.1	+	+	+	+	0	0	0
	0.01	0	+	+	+	0	0	0
		100.6°	103.2°	103.8°	104.4°	104.2°		
Normal control	0.1	±	++	+++	++++	+++++	+++++	Found dead
	0.01	0	+	++	+++	+++++	+++++	
	0.001	0	0	+	++	++++	+++++	
	0.0001	0	0	0	+	+++	++++	
	0.00001	0	0	0	0	++	+++	
	0.000001	0	0	0	0	+	++	

In this and other tables the plus signs represent the relative size and appearance of the lesions. + and ++ indicate swelling and congestion and possible slight edema; +++, ++++, and +++++ represent increasingly larger areas with marked hemorrhage, edema, and necrosis.

oculation of recovered spontaneous or experimental pox rabbits associated with the development of local or generalized lesions other than a slight cutaneous thickening, or in the lower dilutions of virus a small slightly congested cutaneous swelling which persisted for 1 to 4 days. With unfiltered virus emulsions, there were occasional instances of slight transient edema and of slight superficial necrosis. Few of the

lesions attained a diameter of a centimeter and the majority were smaller. By the end of a week and usually before this time the skin appeared normal, while in the normal controls, on the other hand, the cutaneous lesions were still progressing actively. The results in the intravenously and intratesticularly injected rabbits were likewise negative.

The results of these experiments show that recovery from either the spontaneous or the experimental infection with pox renders the rabbit refractory to an injection of pox virus. The condition is apparently a true immunity since, as will later be shown (3), the sera of recovered rabbits possessed complete neutralizing properties when combined with active virus.

Such questions as the time of development of immunity, its completeness with respect to different tissues, and its duration could not be investigated. There was some clinical evidence to suggest that the immunity develops by stages or degrees. In both the spontaneous and experimental disease it was occasionally noted that reactivation and reinduration of skin papules occurred after healing was well advanced, and in both conditions, but more frequently in the experimental infection, new lesions developed when older ones were regressing. True relapse, however, was not observed.

Crossed Inoculation Experiments with Immune Rabbits

The comparisons of pox with other viruses comprised first the inoculation of pox recovered rabbits with the viruses of virus III disease, with dermo- and neurovaccine, and with the virus of infectious myxoma of rabbits, and second the inoculation of virus III and vaccinia recovered rabbits with pox virus.[1] For clarity and in order to save space, the results are presented in three sections in which the respective experiments dealing with virus III, infectious myxoma, and vaccinia are discussed and in this order.

[1] Experiments with rabbit pox and the Shope fibroma virus were kindly carried out by Dr. Carl TenBroeck and will be reported by him. Suffice it to say here that there was apparently no specific relationship between the two viruses.

Virus III Experiments

Pox Recovered Rabbits.—It was found that pox recovered rabbits were susceptible to inoculation with virus III.

Experiments were carried out on 6 rabbits; 2 had recovered from the spontaneous disease noted 25 and 30 days previously and 1 of these animals had also been injected with filtered pox virus 15 days previously with negative results. 4 rabbits had recovered from the experimental disease, the inoculations of which had been made 31, 32, 37, and 52 days previously by the intratesticular, intradermal, or intranasal routes. All 6 rabbits were injected intradermally at 1 or 2 sites with full strength virus III tissue emulsion in 0.3 cc. doses. 2 of the rabbits were also injected intratesticularly with 1.0 cc. doses. The animals were distributed in 3 experiments each of which included similarly inoculated normal rabbits.

The results were uniformly positive, that is, the reactions which developed in the pox recovered rabbits were comparable to those of the control animals. At all 9 sites of intradermal injection congested cutaneous swellings developed on the 1st or 2nd day, increased in size for 3 or 4 days, and then subsided. Some edema and slight hemorrhage was noted in 2 and superficial necrosis in 3 lesions. An orchitis of moderate grade, comparable to that observed in normal animals, developed in the 2 rabbits inoculated intratesticularly. Fever was recorded in 1 of these rabbits and in another inoculated intradermally.

Virus III Recovered Rabbits.—The results of 4 tests showed that rabbits which had previously been inoculated with virus III were not refractory to inoculation with pox virus and that the resulting infection was similar to that developed by normal rabbits.

One rabbit inoculated intratesticularly with virus III 14 days previously was injected intratesticularly with 0.4 cc. doses of pox tissue-virus filtrate and intradermally with 0.1 cc. doses of 4 dilutions of the filtrate ranging from 1:1 to 1:1,000. 3 rabbits inoculated in both testicles and intradermally with virus III 14, 21, and 23 days previously were injected intradermally with 0.2 cc. doses of unfiltered tissue-pox virus in 6 dilutions ranging from 1:10 to 1:1,000,000.

The local lesions which developed at each injection site were indistinguishable from those of the normal controls as regards time of development, appearance, and course. In addition, there developed in each rabbit a typical febrile reaction, a characteristic generalized maculopapular eruption of the skin, and well defined signs of eye and nasal involvement.

The results on recovered pox and virus III rabbits inoculated with the heterologous virus indicate that the two viruses are neither identical nor do they possess any immunological relationships one with the other.

Infectious Myxoma Experiment

Rabbits which had recovered from rabbit pox were as susceptible to the virus of infectious myxoma as normal rabbits.

The experiment which demonstrated this fact was carried out on a group of 12 rabbits of which 8 were recovered pox rabbits, 4 spontaneous and 4 experimental cases, and 4 were normal controls. The symptoms of the spontaneous cases had been observed 3 weeks to 2 months previously while the experimental cases had been inoculated 29 to 33 days previously. Myxoma virus was injected intradermally in doses of 0.25 cc.[2]

The reaction of the pox recovered animals to the myxoma virus was indistinguishable from that of the normal controls as measured by the length of the incubation period, the character of the ensuing lesions, and the time of death. All the animals were moribund 8 days after inoculation and were killed.

The results of the experiment indicate that the virus of rabbit pox is not identical with that of infectious myxoma and that there is no immunological relationship between them. Since infectious myxoma is an invariably fatal condition in normal rabbits, experiments with myxoma immune rabbits or with immune serum could not be made.

Vaccine Virus Experiments

Pox Recovered Rabbits.—Consistently negative results with one possible exception were obtained on 5 recovered pox rabbits injected with full strength culture vaccine virus.

One animal had been inoculated intranasally with pox virus 31 days previously and the others were spontaneous cases whose symptoms had been noted 4 to 6 weeks previously. The duration of recovery was estimated at 2 to 5 weeks. 4 male rabbits were injected intratesticularly with 0.5 or 1.0 cc. doses and intradermally at several sites with 0.2 or 0.3 cc.; a doe was injected intradermally. In 3 cases, virus was applied to the scarified corneas and in 2 cases to scarified skin areas. The rabbits were distributed in 3 experiments which included similarly inoculated normal rabbits.

During the first 48 hours after injection, the color of the skin about the injection site or along the lines of scarification was pale yellow or yellowish pink, but there was no cutaneous thickening or other change and by the 3rd day the skin had a normal appearance. No palpable alteration of the injected testicles was detected. In 1 animal in which virus had been dropped in the eyes, slight conjunctival and pericorneal injection and slight clouding of the corneas developed on the 3rd day, persisted for 2 to 3 days and then subsided. No reaction was observed in the eyes

[2] We are indebted to Dr. T. M. Rivers for the inoculation of myxoma virus and for the subsequent clinical observations.

of the other 2 pox immune rabbits given a conjunctival instillation of vaccine virus. In the normal rabbits injected with culture vaccine virus by the same routes, typical vaccinal reactions developed in each injection site.

Vaccinia Recovered (Culture Virus) Rabbits.—Variable results were obtained on 19 vaccinia recovered rabbits injected with pox virus.

The vaccinal infection was produced by culture vaccine virus injected by the intradermal or intratesticular route; in 16 animals the interval between the 2 injections was 18 to 32 days and in 3 it was 45 to 68 days. For 11 rabbits Berkefeld filtrates of pox tissue emulsions were employed and for 8 unfiltered emulsions. 13 animals were injected intradermally at 56 sites with 0.1 to 0.25 cc. doses of full strength and diluted emulsions. 2 animals were given intratesticular injections of 0.5 or 1.0 cc.; 3 rabbits were injected intravenously with 1.0 cc. and 2 of them also received intradermal injections while both corneas of the third animal were scarified; in 1 animal virus was dropped on the scarified corneas. The rabbits were distributed in 7 experiments which included normal rabbits similarly injected.

The results of the experiments were as follows: In the cases in which pox virus *filtrates* were used, no local reaction was detected in the injected testicles or eyes, but in 3 of the 7 cases injected intradermally there were peculiar cutaneous changes at the sites in which full strength virus and dilutions of 1:10 and 1:100 had been used. On the day after injection, there was a small circular diffuse swelling, the area was resistant and the skin quite congested. During the following 2 or 3 days the lesions increased in size to a diameter of half a centimeter or more; the induration and congestion were more pronounced; and some edema developed. Hemorrhage and necrosis were not observed. By the 4th or 5th day the swelling had subsided and the skin appeared normal. No generalized lesions were observed in 8 of the 11 rabbits injected with filtrates, but in 3 animals cutaneous papules developed.

In 1 rabbit injected intradermally, 2 small pink papules developed in the shaved skin of the injection area on the 4th day and persisted for 48 hours. In a rabbit inoculated intratesticularly a papule appeared in the shaved skin of the body on the 4th day and persisted for 3 days. The 3rd instance was in a rabbit inoculated intravenously; 2 small and 3 medium sized papules developed in a shaved skin area of the body on the 4th day and persisted for 5 days. Fever and other evidence of constitutional disturbance were not observed. It may be pointed out that there was no essential difference in the periods of time between the 1st injection of vaccine virus and the 2nd of pox virus in the case of the 3 rabbits with cutaneous papules and the 8 rabbits in which none was observed. In all 11 animals the interval was 18 to 32 days.

In the tests with the more potent *unfiltered* pox virus which were limited to intradermal injections, the local reaction resembled the occasional lesion observed with filtrates, but it was more pronounced. 8 vaccine immune rabbits distributed in 3 experiments were injected at 28 sites with doses of 0.25 cc. full strength virus.

TABLE II

Results of the Injection of Unfiltered Rabbit Pox Virus in Rabbits Recovered from a Vaccinal Infection (Culture Virus)

Rabbit	Dosage Intradermal injection	Days after inoculation (Rectal temperature (°F.))							
		1	2	3	4	5	6	7	8
	cc.	102.3°	102.6°	103.0°	103.3°		103.6°	102.6°	
A	0.25	+	++	++	Indurated flat yellowish pink swellings with whitish centers	+	>	>	0
	"	+	++	++		+	>	>	0
	"	+	++	++		+	>	>	0
	"	+	++	++		+	>	>	0
		102.6°	102.6°	103.0°	103.0°		103.6°	102.6°	
B	"	+	++	++		++	>	>	0
	"	+	++	++	As above	++	>	>	0
	"	+	++	++		++	>	>	0
	"	+	++	++		++	>	>	0
		102.2°	102.8°	102.5°	102.9°		103.6°	103.0°	
C	"	++	++	++	+++	++	>	>	0
	"	++	++	++	+++	++	>	>	0
	"	++	++	++	+++	++	>	>	0
	"	+++	++	++	++	++	>	>	0
	0.25 cc. of dilution	102.0°	103.9°	103.0°	104.5°		101.9°	99.6°	
Normal control	0.1	0	+	++++	+++++	+++++	<		Found dead
	0.01	0	+	+++	++++	++++	<	Large swollen areas with edema, hemorrhage, and necrosis	
	0.001	0	±	++	++++	++++	<		
	0.0001	0	0	+	+++	++++	<		
	0.00001	0	0	+	+++	+++	<		
	0.000001	0	0	0	++	+++	<		
						6th day—macular papular rash on body and ears; lip papules; bilateral orchitis; profuse nasal discharge; popliteal adenitis; weak and ill			

Signs + to +++++ as in Table I. < = lesions progressing. > = lesions regressing.

3 typical protocols are summarized in Table II. It will be noted first that in the recovered vaccinia rabbits a cutaneous reaction was noticeable within 24 hours, whereas in the normal control rabbit it did not occur until the 2nd day. The normal rabbit, however, was injected with smaller doses, that is, dilutions of virus ranging from 1:10 to 1:1,000,000. In the vaccine immunes, the reaction comprised small red or yellowish red firm cutaneous swellings which increased rapidly in size and by the 3rd or 4th day were 3 or 4 cm. in diameter. The margins of the lesions were not sharply demarcated but faded out into normal skin. On the 2nd and 3rd days some edema and a few small areas of hemorrhage and superficial necrosis had developed in certain lesions. The height of the reaction was usually reached on the 3rd or 4th day. Regression then began and by the 7th or 8th day the lesions comprised firm cutaneous thickenings usually with a scaling surface. A febrile reaction in 3 of the 8 rabbits was first noted on the 2nd and 3rd days. No generalized lesions were observed, none of the animals appeared ill, and there were no fatalities.

The pronounced reactions of the normal control rabbits to both Berkefeld filtered and unfiltered pox tissue emulsions were entirely typical. The protocol of the normal control in Table II gives a good idea of the comparative size and course of the lesions produced by various dilutions of virus. The lesions comprised well defined congested cutaneous swellings which increased very rapidly in size and in which marked hemorrhage, edema, and necrosis were conspicuous features. Fever at some time was regularly observed and generalized manifestations were frequent.

The results of these two groups of experiments show first, the highly refractory state of recovered rabbit pox animals to culture vaccine virus injected intratesticularly, intradermally, and by the scarified skin and cornea routes, and second that recovered vaccinia rabbits are highly but not absolutely protected against inoculation with pox virus injected intradermally, intravenously, or intratesticularly. The reaction of the vaccine immune animals to intradermal injections of pox virus appeared to be of an accelerated or hypersensitive type in so far as the early development and the appearance of the local lesions was concerned, but in addition some lesions continued to be active and persisted for upwards of a week although in no case was the result comparable to that obtained in normal rabbits. A few instances of fever and a minor papular eruption of the skin were observed, but none of the rabbits became ill and none died. These findings point to some relationship between pox virus and vaccine (culture) virus.

Neurovaccine Recovered Rabbits.—Crossed inoculation of rabbits recovered from neurovaccinia had to be limited for various reasons to a single experiment (Table III). The experiment also included rabbits which had recovered from infection

TABLE III

The Reaction of Rabbits Immune to Dermo- (Culture) Vaccine Virus, Neurovaccine Virus, and Rabbit Pox Virus to the Intradermal Inoculation of These Viruses

Rabbit	0.3 cc. at 3 sites Rabbit pox virus Days after inoculation							0.3 cc. at 3 sites Neurovaccine virus Days after inoculation							0.3 cc. at 3 sites Culture vaccine virus Days after inoculation						
	1	2	3	4	5	6	7	1	2	3	4	5	6	7	1	2	3	4	5	6	7
Culture Vaccine Immune																					
A	+	+++	++++ E	++++ EHN	<	>	+++	+	++ E	++	+++ E	>	>	+	0	+	0	0	0	0	0
B	+	+++	++++ E	++++ EHN	<	>	+++	0	++	++	+++ N	<	>	+	0	±	0	0	0	0	0
C	+	+++	++++ E	+++ EH	<	>	++	0	++	++	++ E	+++ HN	>	++	0	±	0	0	0	0	0
Neurovaccine Immune																					
A	+	++ E	++	+++ E	+++ N	<	+	0	++ E	++	++ N	>	>	±	0	+	±	0	0	0	0
B	0	+	+	++	+++ N	<	+	0	+++ N	+++	+++	>	>	+	0	0	0	0	0	0	0
C	+	+++ E	+++	++++ EHN	++++	++++	++++	0	++ E	++	++	>	>	++	0	±	0	0	0	0	0
D	+	++	++	++ E	+++ E	<	+	0	++	++	++ E	>	>	0	0	+	±	0	0	0	0

Rabbit Pox Immune

A	+	++	++	+++ E	>	>	>	0	++	++	+ E	++	>	+	0	+	0	0	0	0	0
B	0	+	+	++ E	>	±	±	0	++	++	++ EN	>	>	+	0	±	0	0	0	0	0
C	0	±	+	+	>	0	0	0	+	++	+++ EN	+++	>	+	0	+	0	0	0	0	0
D	0	+	+	+	>	0	0	0	+	+	++	+++	>	+	0	±	0	0	0	0	0

Normal Control

A	++	++++ EH	++++* EHN	<	<	<	+++++	+	+++ E	+++	+++ E	+++	>	+	+	++ E	++	>	+	±	±
B	++	++++ EH	++++* EHN	<	<	<	+++++	+	++	+++	+++ EHN	+++	>	+	+	++ E	++	>	+	0	0

Signs + to +++++ as in Tables I and II. < = lesions progressing; > = lesions regressing. E = edema; H = hemorrhage; N = necrosis. * = confluent lesions.

with culture vaccine virus or experimental pox, so that the results supplement those discussed in the preceding section. The reinoculation of the three viruses was carried out on 4 rabbits 66 to 75 days after inoculation with neurovaccine virus, on 3 rabbits 66 to 74 days after inoculation with culture vaccine virus, and on 4 rabbits 66 to 136 days after inoculation with pox virus. There were 2 normal controls. In the case of each virus pooled emulsions were prepared from the testicles of 3 rabbits with an acute marked orchitis. Unfiltered full strength emulsions were used. 3 intradermal 0.3 cc. doses of each virus were injected in each rabbit, the pox virus on the right side of the body, the culture vaccine virus on the upper left, and the neurovaccine virus on the lower left side. Since the three reactions to each virus in individual animals were quite similar, they have been represented by one sign in the summary of results in Table III.

The results in the normal animals will first be described. There were definite differences in the character of the reaction to the three viruses as shown particularly by the rate of development of the lesions, their size and duration, and the presence and degree of edema, hemorrhage, and necrosis. The reaction to pox virus was the most severe and that to culture vaccine the least, while the response to neurovaccine occupied an intermediate position. The pox lesions were well developed on the 2nd day, they soon became very large and confluent, and were still active at the end of a week (Fig. 1). Pronounced edema, hemorrhage, and necrosis were present in all of them. The neurovaccine lesions did not develop as soon or as rapidly as the pox lesions and by the 6th and 7th days were definitely regressing (Fig. 2). They preserved a nodular, circumscribed character and were not confluent. Edema, hemorrhage, and necrosis developed but to a considerably less degree than in the pox lesions. In the case of culture vaccine virus, the lesions were comparatively small, edema was not marked, hemorrhage, and necrosis were comparatively limited, and healing was practically complete by the end of a week (Fig. 2).

The intradermal reaction of 3 *vaccinia immune* rabbits to a 2nd injection of culture virus was practically negative. There were small nodular lesions on the 2nd day which did not persist (Table III). In the photograph taken on the 6th day, the injected areas show only a luxuriant hair growth (Fig. 4). With neurovaccine and pox virus, on the other hand, the results were positive, but the pox lesions were much the larger and showed more edema, hemorrhage, and necrosis (Figs. 3 and 4). In both cases, however, the lesions were less pronounced than those of the normal controls and regression was well under way by the end of the week.

In the case of 4 rabbits *recovered from neurovaccinia* and reinoculated with this virus, definite cutaneous lesions developed in all 12 sites of injection (Table III). Nodular congested and somewhat edematous swellings were noted on the 2nd day. They increased in size for 2 or 3 days, some necrosis developed, and then definite regression began; the appearance on the 6th day is shown in Fig. 6. The general character and degree of the reaction indicated a partial but not a complete refractory state to reinoculation of neurovaccine. The reaction of these animals to intradermal injection of culture vaccine was practically negative. There were 4

instances of small transitory nodular swellings, but by the 4th day the skin in all 12 injection sites appeared normal and continued so (Fig. 6). In contrast to these results the intradermal injection of pox virus uniformly produced well marked positive reactions with edema, hemorrhage, and necrosis, as shown in the photograph in Fig. 5 taken on the 6th day. The lesions were more pronounced than those of the homologous neurovaccine but were less marked than those in the normal controls and in the culture vaccine immune animals.

The reaction of 2 of the 4 *pox immune* rabbits to reinoculation with pox virus was considered negative (Table III); the interval between the 1st and 2nd inoculations was 66 days. The slight lesions which developed comprised small flat superficial areas of thickening which developed on the 2nd and 3rd day, the skin was faintly reddened and slight transient edema developed in some instances, but there was no hemorrhage or necrosis. A week after injection the skin was but slightly thickened and discolored (Fig. 7). A similar reaction was observed in 1 of 2 other pox immunes inoculated 136 days previously, but in the 2nd of these older immune animals a peculiar response was obtained which was classified as positive (Table III). The day after injection the skin about the injection sites was somewhat thickened and congested; the areas then enlarged in extent but only slightly in depth, and gradually the skin became cherry red and then a deep reddish brown color. There was a slight transient edema but no necrosis. The margins of the lesions were well defined. Regression began on the 5th day and healing was soon accomplished. The reaction of all 4 pox immune rabbits to injection with culture vaccine virus was negative; the slight skin changes on the 2nd day did not persist thereafter. In the case of neurovaccine virus, the reaction was considered to be partially positive (Table III) but it was clearly much less marked than that which developed in the normal controls. The lesions had the same general appearance as those in the normal rabbits but they developed a day later, did not attain as large a size, and their circumscribed nodular character was more pronounced. Their appearance on the 6th day when regression had begun is seen in Fig. 8. There was no obvious difference in the neurovaccine lesions which developed in the more recent as compared with the older pox immune animals.

The results of this comparative experiment supplement the suggestions regarding the nature of pox virus which arose from the observations on the experiments with culture vaccine virus described in the preceding section. In the first place, the modified reaction to intradermal injection of the viruses of pox, culture vaccinia, and neurovaccinia in rabbits immune to one of them indicates some common relationship. Rabbits which had recovered from pox, culture vaccinia, or neurovaccinia were completely refractory to culture vaccine. Culture vaccinia recovered rabbits were partially refractory to pox and were even more so to neurovaccine virus. Neurovaccine recovered

rabbits were partially refractory to neurovaccine but little more than were the culture vaccine immunes; they were somewhat refractory to pox virus and on the whole more so than were the culture vaccine immunes. Pox recovered rabbits were with one possible exception refractory to reinoculation with pox. They showed a partial refractoriness to the neurovaccine which, however, was probably less effective than that of neurovaccine immunes to reinoculation with neurovaccine, but which was more effective than the refractory state of the neurovaccine immunes to pox virus. From the standpoint of a refractory state to intradermal injections of all three viruses, the rabbits which had recovered from pox were more efficiently protected than were those which had recovered from neurovaccinia or culture vaccinia. And on the whole, the neurovaccine immune rabbits were more efficiently protected than the culture vaccine immunes. In this connection, it should be pointed out that the positive reactions of culture vaccine recovered rabbits to injection of pox virus in the present experiment were much more pronounced than those of previous experiments. It is not unlikely that the reason for the present result is connected with the longer period between vaccination and the pox injection and a consequent diminution of immunity.

In the second place, while the cutaneous lesions of the three viruses produced by intradermal injection in normal animals had a general resemblance one to another, there were well defined and consistent differences between them with respect to the rate and degree of development, the degree of hemorrhage, edema, and necrosis, the duration of activity, and the initiation and rate of healing. Differences of the same order were also observed in the lesions produced in the recovered (immune) animals of the experiment. From this standpoint of severity of reaction, pox virus was by far the most potent and culture vaccine the least while neurovaccine was less potent than pox virus but much more potent than culture vaccine. The rapid invasive growth of pox lesions with confluence of adjacent lesions was especially marked. The lesions of neurovaccine virus, on the other hand, were circumscribed and nodular, and the degree of edema, hemorrhage, and necrosis was very much less than in the lesions of pox virus. Although these differences were regularly observed, it must be remembered that the opportunity for studying neurovaccinia was limited and that

a greater experience might have shown them to be less pronounced or less uniform. It is difficult to say upon the basis of such a criterion as the general character of cutaneous lesions how far one is justified in assuming that essential differences between viruses exist. That these three viruses did not produce lesions of similar degree or severity, however, was certain, and from this standpoint it is permissible to conclude that at least they exhibited qualitative differences of pathogenicity.

Exposure of Rabbit Pox, Virus III, and Vaccinia Immune Rabbits to Pox

The exposure of rabbits which had recovered from pox, virus III, or vaccine infections to cases of pox took place in two rooms containing many animals with pronounced clinical symptoms of the disease. The rabbits with the exception of 12 young animals of one experiment were kept in individual cages and all were fed and cared for by the same persons. Before taking up the results on immune rabbits, the findings on normal rabbits exposed to room and cage infection will be presented.

Normal Rabbits.—Exposure experiments with normal animals were limited since this aspect of the work was done in connection with the investigations on the spontaneous disease (4).

Four normal adult rabbits were placed in the rooms at different times. All developed pox. In 3 cases the first clinical manifestations of the infection were noted on the 6th, 11th, and 15th days respectively; one animal was found dead on the 21st day while the others recovered from infections classified as moderately severe. The incubation period of the disease in the 4th animal is not known, but fever, a popliteal adenitis, and a few regressing cutaneous papules were observed on the 26th day.

Six young rabbits, the control animals in an experiment with vaccine immune animals to be described, were kept in the same pens with rabbits with marked clinical pox. All developed the disease; it was of moderate or marked severity in 5 cases and mild in the 6th (Table IV). The earliest lesion was observed on the 5th day. In 2 other animals lesions developed on the 6th, in 1 rabbit on the 9th, in another on the 12th, and in the last animal on the 14th day respectively. The 2 fatalities occurred on the 14th and 21st days. A 3rd animal with marked symptoms became seriously ill and was killed on the 14th day. The mean incubation period of the disease in the 3 adult rabbits was 10.7 days and in the 6 young animals in actual contact with pox cases was 8.7 days.

Pox Recovered Rabbits.—During the 5 months in which most of the work on experimental rabbit pox was carried out, many rabbits which had recovered from

either the experimental or the spontaneous infection were caged in the same rooms with active cases of experimental pox. The duration of exposure ranged from a

TABLE IV

Results of the Cage Exposure to Rabbit Pox of Vaccinated (Culture Vaccine) and Normal Rabbits

Rabbit	Fever		Clinical manifestations			Result
	First record	Highest recorded	First lesion			
	days	*°F.*	*days*			
Vaccinated Rabbits						
1	13	105.5	12	Popliteal adenitis.	Lip papules. Nasal discharge	Recovered
2	11	104.9	14	" "	Diarrhea	"
3	10	105.3	10	" "	Lip papules. Conjunctival discharge	"
4	6	105.6	12	" "	Nasal discharge	"
5	10	104.1	12	" "	Diarrhea	"
6	6	104.1	5	" "	Lip papule	"
Normal Rabbits						
1	12	104.2	12	Popliteal adenitis.	Nasal discharge	Recovered
2	7	105.6	5	" "	Marked general mucocutaneous rash. Diarrhea	Dead 21st day
3	—	103.7	14	" "		Recovered
4	6	106.8	6	" "	Mucocutaneous rash. Nasal discharge	"
5	6	107.0	6	" "	Marked mucocutaneous rash. Nasal discharge. Diarrhea	Dead 14th day
6	6	107.4	9	" "	Marked mucocutaneous rash. Nasal and conjunctival discharge. Keratitis. Diarrhea	Moribund; killed 14th day

few days to 3 months or more. In no instance was there any clinical evidence of reinfection.

Virus III Recovered Rabbits.—When the crossed inoculation and virus-serum neutralization experiments were in progress, rabbits inoculated with virus III were kept in a separate room. At the expiration of this work, 4 virus III immune

animals were transferred to a pox room. All developed clinical pox. In 2 rabbits lesions were first noted on the 10th day and in the others on the 12th and 14th days respectively. 2 of the cases were mild and the animals recovered; the course and outcome of the infection in the others is not known as the animals were killed shortly after the diagnosis was made.

Vaccinia Recovered Rabbits.—There was no clinical evidence of the spread of pox to adult vaccine immune rabbits.

The 19 rabbits previously inoculated with culture vaccine virus and subsequently injected with pox virus were transferred to a pox room at the time of the pox injection. In 4 instances the wire mesh sides of the cages were placed in direct contract with those of rabbits with florid pox. The interval between vaccination and the time of exposure and injection of pox virus was 18 to 32 days or a mean of 23 days in the first group of 11 rabbits and 25 to 68 days or a mean of 39 days for 8 animals. The duration of exposure was variable but in most cases it was 2 or 3 months and in none less than a month.

As soon as it was reasonably certain that under these conditions vaccine immune rabbits did not develop clinical pox, an experiment was carried out in which actual contact of vaccine immunes with active cases of pox was insured.

Two litters comprising 12 English-Lilac hybrids 2½ months of age which had been weaned 12 days previously were procured from a breeding colony in which no evidence of pox had been detected. 6 rabbits were kept in strict isolation during the period of vaccine immunization of their 6 litter mates. Vaccination was carried out by the intradermal injection of 0.2 cc. of culture vaccine tissue emulsion. A typical cutaneous lesion with swelling, congestion, moderate edema, and slight necrosis developed in each rabbit. The first indication of a positive reaction was noted on the 2nd day and by the 7th the lesions were regressing. 16 days after vaccination all 12 rabbits were put in two pens with rabbits showing pronounced pox symptoms. Each pen contained 3 vaccinated and 3 unvaccinated litter mates and 1 rabbit with well marked clinical manifestations of the disease resulting from the intradermal inoculation of a Berkefeld V tissue-virus filtrate derived from the 9th serial filtrate passage of the Xy171 strain of pox virus (1).

The results of the experiment, which are summarized in Table IV, show that contrary to what had been expected on the basis of previous findings, the vaccinated rabbits developed clinical signs of a pox infection. The disease was much less severe, however, than in the unvaccinated control litter mates.

In 5 controls the disease was moderate or marked and the symptoms included 4 instances of a generalized papular rash. Rectal temperatures above 104°F. were recorded, the mean value of the highest readings being 106.4°F. The mean time of the first record of fever was 7.4 days and of the the first lesions of the 5 animals was 7.6 days. There were 2 fatalities on the 14th and 21st days respectively and a 3rd animal whose condition became serious was killed on the 14th day. The 6th control had a mild infection.

All 6 vaccinated rabbits developed mild infections and recovered. Fever was observed in each case but the average of the highest readings was 104.8°F., a value considerably below that for the controls. The mean time of the first record of fever was 9.3 days, that is, 2 days later than the control value. The clinical manifestations included a popliteal adenitis in all animals, papules on the lips in 2 cases, conjunctival secretion in 1, nasal discharge in 2, and a diarrhea in 2 cases respectively. There was no instance of a generalized cutaneous eruption. The mean time at which the first lesions were detected was 11.1 days after exposure, that is 4 days later than for 5 controls, or 2.4 days later if the 6th control is included.[3] Post mortem examination on the 26th day showed enlarged indurated popliteal lymph nodes in each animal, bronchopneumonia with lung papules in 2 cases, and suggestive changes of the liver in a 3rd case. The 3 recovered con-

[3] It was not known at the time this experiment was done that intradermal vaccination of the rabbit with vaccine virus may give rise to generalized manifestations, one of which is a generalized papular eruption of the skin and mucocutaneous borders (5). This information was obtained as a result of the vaccination of the breeding colony in 1933–34, the winter following the pox epidemic and the present investigations on experimental pox. In the light of this information, the question arises whether the lesions observed in the 6 previously vaccinated young rabbits which developed after cage exposure to pox, were manifestations of an acquired pox infection or generalized manifestations of vaccinia. The most convincing evidence against the latter possibility is the time at which the lesions developed in connection with the fact of a positive vaccination reaction. The mean time of development was 27 days after vaccination and all 6 animals had had a positive local reaction. The mean time at which generalized vaccinal lesions were observed in a large group of vaccinated adult rabbits was 6 to 10 days after vaccination, and there were but few cases in adults with a negative vaccination. In the young rabbits of one experiment (mean age 48 days) the great majority of the cases of generalized disease occurred in animals with a positive local reaction and at a mean time of 7 days after vaccination. In another experiment comprising slightly younger rabbits (mean age 38 days) the majority of the comparatively few cases of generalized manifestations which developed after a positive vaccination were detected within the first fortnight. There was also a small group of recently weaned animals in some of which generalized lesions developed at a mean time of 25 days after vaccination, but 90.0 per cent of these rabbits had had a negative vaccination.

trols which were also killed on the 26th day showed a similar condition of the lymph nodes and in 1 animal a patchy pneumonia of uncertain significance.

The results of this experiment show that under certain conditions it is possible for rabbits previously vaccinated with vaccine virus to contract rabbit pox from cage exposure. The clinical manifestations, however, were much less severe than those which developed in the unvaccinated litter mates, and the conclusion that vaccination may afford a marked measure of protection against pox is thus clearly indicated. But the fact that vaccine immune rabbits developed clinical pox even in mild degree raises the question of why a similar result was not obtained in any of the 19 previously vaccinated rabbits which were not only exposed to pox but were also injected with pox virus. It is true that in the one case the animals were in actual contact with pox rabbits while in the other they were exposed to a room infection. In view, however, of the highly contagious nature of the infection the difference between cage and room exposure is probably of little significance.

There were three other respects, however, in which the 6 animals of the cage exposure experiment differed from the other 19 vaccine immune rabbits.

First, the latter animals were injected by one or another route with pox virus at the time they were transferred to the pox rooms. Had any degree of susceptibility to pox existed at this time, one might have expected that the combination of virus inoculation and continuous ample opportunity for room infection would have resulted in the development of pox manifestations in some if not in all cases. In certain of these rabbits as was previously pointed out, the reaction to the injection of pox virus suggested that such a susceptibility did exist, but the manifestations which developed were even of a more minor nature than that observed in the animals of the cage experiment. Perhaps the explanation is concerned with an initial partially incomplete resistant state which was rapidly augmented to complete resistance by the reaction to the injection of pox virus.

Second, there was a difference in the time interval between vaccination and exposure to pox. In the cage exposure experiment this period was 16 days, and in the case of the other 19 animals it varied from 18 to 68 days. In 6 of these rabbits, however, it was 18 to 21 days, and in 7 others 24 to 28 days, so that the actual difference in a comparable number of rabbits was slight. A period of 16 days is generally regarded as being sufficient for the establishment of an efficient vaccinal immunity to reinoculation with certain exceptions such as a delayed corneal immunity of individual animals.

Third, there was an age difference. The 19 vaccinated rabbits which did not develop clinical pox after pox inoculation and pox exposure were mature animals approximately 6 months of age or older. The 6 vaccinated rabbits which developed a mild but definite pox infection after cage exposure were recently weaned animals 2½ months of age at the time of vaccination. Comparatively little is known regarding the influence of age on the reaction of the rabbit to experimental disease, but in the case of syphilis, for example, young rabbits have been regarded as being particularly susceptible and an analogous condition exists with respect to tuberculosis.[4] Recent experience with the vaccination of a rabbit breeding colony has shown that the reaction of animals to intradermal injections of vaccine virus differs in different age groups (5). Several years' observation on this breeding colony has emphasized the fact that one of the critical periods in the life of the rabbit is that of weaning. This period during which the animal must adapt itself to a self-sustaining existence is not infrequently characterized by evidence of a disturbed economy, as for example, an increase in the incidence of gastrointestinal disorders or of such infections as snuffles. In the pox epidemic, there was a very high mortality among animals less than 14 weeks of age and the highest rate occurred among those 4 to 8 weeks old (4). The complex physiological adjustments which take place during the weaning period may impose such a strain upon the animal's capacities that the responses to various conditions are quite different from those which occur at other ages.

In the particular observations under discussion there was no doubt that vaccination in young recently weaned rabbits did not afford the degree of protection against pox that was exhibited by mature vaccinated animals. It is possible that the natural resistance of young rabbits to pox is so much less than that of older animals that the mechanism of vaccinal immunity does not afford a comparable protection. An alternative explanation concerns a possible deficiency in the quality of the vaccinal immunity of young rabbits or a delay in its effective functioning. In this connection one must consider the possibility that the tissues of the portal of entry, presumably those of the respiratory tract in the present case, do not develop a vaccinal immunity effective against pox to the same degree or at the same rate as do other tissues such as the skin.

Reaction of the Calf to Inoculation of Pox and Vaccine Viruses

The comparisons between rabbit pox and vaccinia included observations on the cutaneous reaction of a calf to the several viruses applied to the scarified skin.

The skin on both sides of the body was shaved and scarified immediately before inoculation. On the right side 1.0 cc. unfiltered pox virus tissue emulsion was rubbed into the prepared area. On the left side 1.0 cc. each of culture vaccine

[4] Personal communication from Dr. J. H. Geiger.

virus, of the New York City Board of Health vaccine, and of a tissue emulsion of neurovaccine virus were rubbed into three areas separated by broad strips of unshaven skin. 2 rabbits were similarly inoculated with the four viruses in 0.25 cc. doses and in addition, the same amount of each virus was inoculated on 1 each of 4 rabbits by the same route of cutaneous scarification. Finally, 2 rabbits were injected in both testicles with 0.5 cc. of pox virus for the routine passage of the strain; virus was also applied to the scarified skin.

The results on the calf showed the development of a positive reaction to each virus but the pox lesions were considerably larger and much more persistent than those of the three specimens of vaccine virus. The lesions of the Board of Health vaccine were next in scale while those of neurovaccine and culture vaccine were less pronounced.

During the first 2 days the lines of scarification in all the vaccine areas were congested and prominent while those in the pox area were barely perceptible. On the 3rd day, however, several small papules were noted in the pox, the neurovaccine, and the Board of Health vaccine sites and on the 4th day in the culture vaccine area. These lesions and those which subsequently developed were confined to the lines of scarification. By the 5th and 6th days well developed lesions were present. The predominating type was a papule, but in some instances vesicles were forming and pustules were later observed. And by this time well defined differences in the grade or severity of the 4 lesions were apparent. The pox lesions comprised comparatively large papules and umbilicated vesicles in which hemorrhage and necrosis were conspicuous and in addition some lesions were edematous. The majority were discrete but some were confluent and crusts were forming. Of the vaccinia lesions those produced by the Board of Health virus were the largest but they were less numerous than the pox lesions and the majority were much smaller. Furthermore, there was much less hemorrhage and necrosis, comparatively little umbilication, and no edema. Crusts were beginning. There were more neurovaccine than Board of Health virus lesions, but they were much smaller and more superficial. Slight hemorrhage, necrosis, and crusting were present but no edema. In the case of culture vaccine virus, the reaction was comparatively slight. A few small papules had developed and there was a suggestion of beginning necrosis, hemorrhage, and vesicle formation but the lesions were much less pronounced than those of the other viruses. These differences in severity or grade of reaction continued to be maintained and were especially striking a week after inoculation as is shown by the photographs in Figs. 9 and 10. By this time umbilication and crusting of the pox lesions were particularly conspicuous.

By the 10th day the few culture vaccine papules had shown rapid regression and were almost healed. Those of neurovaccine virus had also shown marked regression; they now consisted principally of superficial fibrous thickenings of the

skin covered by thin blackish partially detached crusts. The lesions of the Board of Health vaccine virus were beginning to heal. There were 3 or 4 comparatively large and still resistant lesions with slight umbilication and thin dry reddish black scabs; the others were small and dry with flaky yellowish crusts. The pox lesions were just beginning to regress; their size which was quite uniform was approximately the same as that of the largest Board of Health vaccine lesions. Practically all were umbilicated, the large central depressed portion being covered with a thick tenacious black or reddish black crust. On the 15th day when the experiment was terminated, the culture vaccine area was practically negative. All that remained of the neurovaccine lesions were a few small dry scabs covering slight cutaneous thickenings. The smaller lesions of the Board of Health vaccine were also healed; the few larger ones could still be detected but rapid resolution and healing were evident. The pox lesions were definitely regressing but they were still large and prominent. Post mortem examination of the calf revealed no gross abnormalities of the viscera.

The results on the rabbits inoculated with the same specimens of viruses used for the calf are of interest from a comparative standpoint.

Two rabbits were injected intratesticularly and on a scarified skin area with pox virus. Both developed fever and a typical orchitis with scrotal edema. One died on the 5th day without developing cutaneous lesions either in the inoculated area or elsewhere. The other animal developed a papular skin eruption on the 4th day which was not confined to the lines of scarification; recovery eventually took place. This case was one of the rare instances in which intratesticular injection of pox virus was not associated with a fatal outcome (1). It is probable that the potency of this particular specimen of virus was lower than usual.

Each of the four viruses was used for the inoculation of a rabbit by the scarified skin route and in addition, 2 rabbits were inoculated with all four viruses as was done in the case of the calf. In each injection area a well marked reaction developed. Speaking generally, the most marked reaction was observed with pox virus, next in order was that of neurovaccine, while those of the culture vaccine and of the Board of Health vaccine virus were less pronounced. On the day following inoculation the skin along the lines of scarification was swollen and congested. By the 3rd day this change was conspicuous and in addition small papules were developing in the scarifications. On the 4th and 5th days definite differences in degree of the four reactions became apparent. The pox lesions comprised broad bands or welts of swollen hemorrhagic edematous skin with central lines or streaks of necrosis covered by black crusts. Papules of the same character, that is with hemorrhage, edema, and necrosis, were present not only in these areas, but in the shaved skin outside them. The neurovaccine lesions were similar in appearance but the degree of swelling, hemorrhage, and edema was less marked and the necrosis was more superficial and less extensive. In the case of both the Board of Health and the culture vaccine lesions, the skin along the scarified lines was very much less swollen and congested than with

either the pox or the neurovaccine lesions. There was an accumulation of fine dry yellowish crusts along the lines which was especially abundant in the Board of Health vaccine lesions, and in addition there were many discrete and confluent small papules, the majority of which had yellowish or yellowish red centers covered with thin dry crusts.

A week after inoculation the pox lesions for the most part still appeared active although some of the more superficial portions were beginning to heal. Areas of skin between and beyond the scarifications had become edematous and hemorrhagic and here there was no indication of regression. In the rabbit inoculated only with pox virus, papules in distant skin areas and of the mucocutaneous borders of the lips had developed, together with a nodular orchitis and a popliteal adenitis. Similar lesions were observed in both rabbits inoculated with pox and the three vaccine viruses, but not in the rabbits inoculated only with the respective vaccine viruses. In contrast to the continued activity of the pox lesions a week after inoculation, the lesions of the three vaccine viruses had regressed considerably, particularly in the case of the Board of Health and culture vaccines. By the 10th day healing of the pox lesions was well under way.

The reactions of the calf and the rabbit to pox virus, on the one hand, and to the three vaccinia viruses, on the other, were of the same order as far as the size, general character, and course of the local lesions were concerned, but there were definite species differences with respect to the vaccine viruses. In the case of the calf the reaction to pox virus was more pronounced than any of the reactions to the vaccine viruses. Of the latter, the reaction to the Board of Health vaccine was more pronounced than those of neurovaccine and of culture vaccine. In the case of the rabbit, pox virus again induced the most severe lesions, but those of neurovaccine were more marked than those of the Board of Health and the culture vaccines.

The results of this experiment indicate, as did the others reported in this paper, that a relationship of some sort but not complete identity exists between the virus of rabbit pox, on the one hand, and dermo- and neurovaccine viruses, on the other.

SUMMARY AND CONCLUSIONS

Experiments are reported in which it was shown that rabbits which had recovered from experimental or spontaneous rabbit pox were refractory to inoculation of pox virus injected by various routes, and in addition did not develop clinical manifestations of the disease under conditions of exposure to florid cases of pox.

It was found that pox recovered rabbits were susceptible to inocula-

tion with the virus of virus III disease of rabbits and that virus III recovered rabbits could be successfully inoculated with pox virus. Furthermore, virus III recovered rabbits developed pox when subjected to room exposure in the same manner as did normal rabbits. It thus appears that there is no specific relationship between the two viruses.

Rabbits which had recovered from experimental or spontaneous pox were found to be just as susceptible to inoculation with the virus of infectious myxoma of rabbits as were normal rabbits, a result which demonstrates that there is no specific relationship between these viruses.

Rabbits which had recovered from experimental or spontaneous pox were refractory to inoculation with culture dermovaccine virus, but vaccine recovered rabbits were not completely refractory to inoculation with pox virus. Under conditions of exposure to clinical cases of pox, adult vaccine immune rabbits did not develop clinical manifestations of pox, but young, recently weaned vaccinated rabbits did contract mild but definite clinical pox.

Experimental pox recovered rabbits were partially refractory to inoculation with neurovaccine virus and neurovaccine recovered rabbits were partially refractory to inoculation with pox virus. The refractory condition of the pox immune rabbits appeared to be more pronounced than that of the neurovaccine immunes.

The cutaneous lesions which developed from the intradermal injection of pox, neurovaccine, and culture vaccine viruses showed definite differences with respect to the rate and persistence of active growth, amount of edema, hemorrhage, and necrosis, and the degree of tissue destructiveness. These features were most pronounced in the lesions of pox virus and were least marked in the lesions of culture vaccine virus. The differences were particularly apparent in normal rabbits, but they were also present in the lesions which developed in immune animals.

It was found that the calf was susceptible to inoculation with pox virus applied to a scarified skin area. There were many similarities in the appearance and course of the pox lesions to those resulting from culture vaccine virus, the New York Board of Health vaccine, and neurovaccine virus similarly inoculated. But the pox lesions

were most numerous, much the largest and most destructive, and by far the most persistent while next in order were those of the Board of Health dermovaccine.

The results of these various experiments showed that a close relationship obtains between pox virus, on the one hand, and vaccine virus and neurovaccine virus, on the other, but it cannot be said that pox virus is identical in all respects with either one of these viruses. The findings indicated that the relationship between pox and neurovaccine viruses is closer than that between pox and culture vaccine viruses. Upon the basis of the results observed in culture (dermo) vaccine immune rabbits inoculated with or exposed to pox, it appeared that vaccination with vaccine virus offered a method of protection against rabbit pox.

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EXPLANATION OF PLATES

FIGS. 1 through 8. 6 days after intradermal injection of testicular tissue-virus emulsions. Each virus injected at 3 sites in 0.3 cc. doses.

PLATE 27

FIGS. 1 and 2. Normal control rabbit. Fig. 1. Right side injected with rabbit pox virus: active massive confluent lesions with extensive hemorrhage, edema, and necrosis. Fig. 2. Upper left side injected with dermo- (culture) virus: small lesions practically healed. Lower left side injected with neurovaccine virus: well advanced regression of moderate sized nodular lesions; subsidence of edema and hemorrhage and healing of necrotic areas.

FIGS. 3 and 4. Dermo- (culture) vaccine immune rabbit. Fig. 3. Right side injected with rabbit pox virus: regressing large necrotic lesions. Fig. 4. Upper left side injected with dermo- (culture) vaccine virus: negative; luxuriant growth of hair. Lower left side injected with neurovaccine virus: marked regression of moderate sized lesions.

FIGS. 5 and 6. Neurovaccine immune rabbit. Fig. 5. Right side injected with rabbit pox virus: regressing moderate sized lesions; hemorrhage, edema, and necrosis still present. Fig. 6. Upper left side injected with dermo- (cul-

ture) vaccine virus: negative. Lower left side injected with neurovaccine virus: regressing moderate sized lesions; some edema, congestion, and slight necrosis still present.

PLATE 28

FIGS. 7 and 8. Rabbit pox immune rabbit. Fig. 7. Right side injected with rabbit pox virus: negative; small superficial cutaneous thickenings. Fig. 8. Upper left side injected with dermo- (culture) vaccine virus: negative. Lower left side injected with neurovaccine virus: partially positive; regressing small nodules.

FIG. 9. Cutaneous reaction of a calf 8 days after the application of rabbit pox virus to a scarified area on the right side of the body.

FIG. 10. Cutaneous reactions of a calf 8 days after the application of vaccine viruses to scarified areas on the left side of the body; same animal as in Fig. 9. Left area, New York City Board of Health dermovaccine; middle area, dermo- (culture) vaccine virus; right area, neurovaccine virus.

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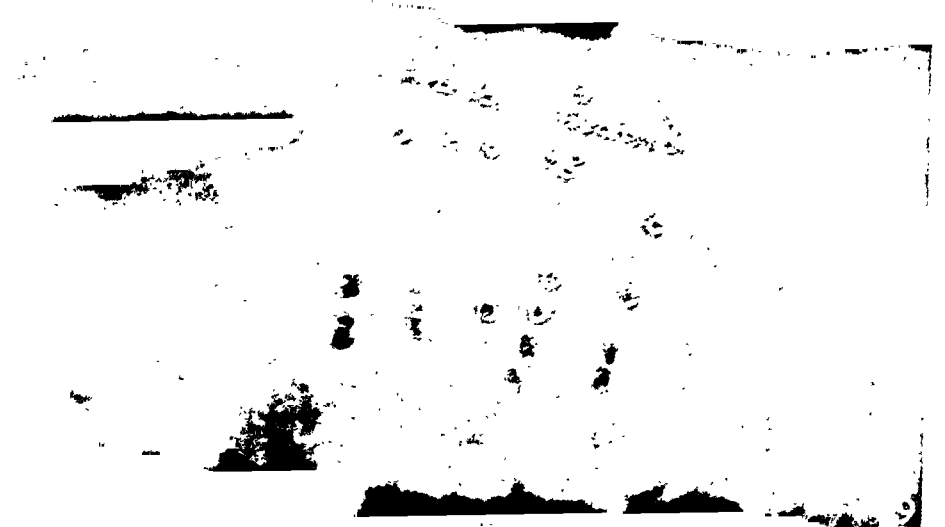

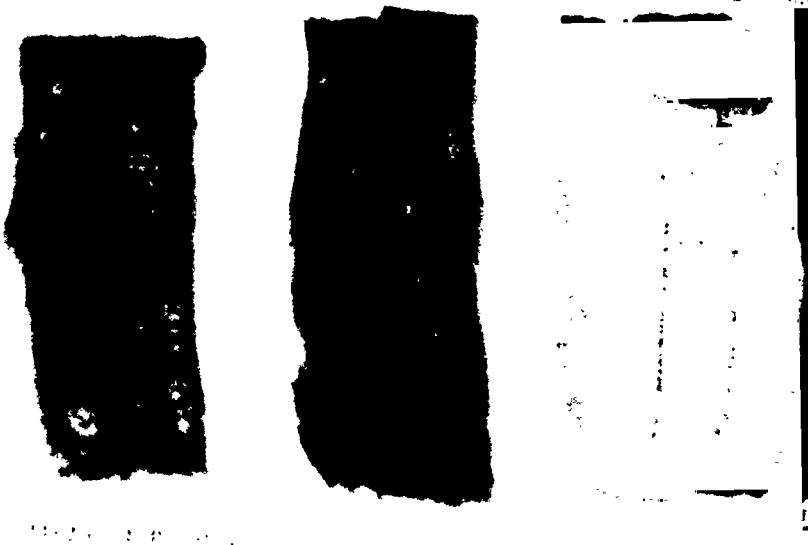

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STUDIES ON THE ETIOLOGY OF RABBIT POX

IV. Tests on the Relation of Rabbit Pox Virus to Other Viruses by Serum Neutralization Experiments

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Plate 29

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The preceding paper contains the results of various reinoculation experiments in which the viruses of rabbit pox, virus III disease, infectious myxoma of rabbits, culture (dermo) vaccinia, and neurovaccinia were compared (1). The findings with respect to the reaction of recovered rabbits to exposure to clinical cases of pox, together with the observations on a calf inoculated with pox, two specimens of vaccine virus, and neurovaccine were also reported. The present paper is concerned with the results of the serum neutralization tests in which various combinations of the viruses and the immune sera of pox, vaccinia, neurovaccinia, and virus III were studied.

Materials and Methods

The various virus emulsions were prepared from tissues obtained from cases of acute orchitis produced by intratesticular injection of tissue-virus. The testicles were ground with alundum and Locke's solution to make a suspension of 10 to 20 per cent concentration by weight. Gross particles were removed by low speed centrifugation. The emulsions were kept in the ice box while their bacterial sterility was tested.

The immune sera were obtained from rabbits inoculated 17 to 134 days previously or in the case of spontaneous pox, a fortnight or more after all signs of the disease had healed. Each specimen was kept in sealed tubes in the ice box. The sterility of each serum was tested before tubing and before use.

The tests were carried out in the usual manner. Desired dilutions of virus or serum were made with Locke's solution. Equal parts of tissue-virus emulsion and of serum were thoroughly mixed in small sterile Petri dishes and allowed to stand at room temperature 1½ to 2½ hours; 0.2 cc. of the mixture was then injected

in normal adult rabbits intradermally on the shaved skin of the side of the body. In certain experiments, it was necessary to inject mixtures of more than one virus in the same animal. The combinations of a different immune sera with a particular virus were injected in the same rabbits. Appropriate control mixtures of virus and normal serum and of virus and the homologous immune serum were made. Each set of tests was done on 2 animals. The animals were examined daily. The results were usually limited to the findings of a week on account of the pronounced disease with its marked constitutional symptoms produced by pox virus (2, 3).

RESULTS AND DISCUSSION

The results of the serum-virus neutralization experiments are presented in the following order: First, the observations on the neutralizing action of pox immune serum against pox virus; second, the findings with respect to virus III immune serum and pox virus; and third, the action of culture (dermo) vaccine and neurovaccine immune sera against pox virus and the action of pox immune serum against culture vaccine and neurovaccine viruses. It seems best to discuss the results of each group of experiments as they are presented. The major interest in the findings centers around the experiments in which pox and vaccine immune sera were compared, for it was found that pox immune serum had a marked neutralizing action against pox virus, vaccine virus, and neurovaccine. Vaccine immune serum had a partial neutralizing power against pox virus and neurovaccine immune serum was more potent in this respect than culture vaccine immune serum. Virus III immune serum, on the other hand, had no neutralizing action against pox virus. Summarized typical protocols of several sets or groups of experiments have been selected for discussion of the results obtained (Tables I to IV).

Pox Immune Serum-Pox Virus Tests.—It was found that pox immune serum possessed marked neutralizing properties against pox virus.

In the first experiments in which pox immune, culture vaccine immune, and virus III immune sera were compared with respect to their neutralizing action against pox virus, the serum-virus mixtures comprised full strength serum plus various virus dilutions up to 1:100,000. Three pox immune sera were tested, two from experimental cases obtained 31 and 33 days after inoculation and one from a spontaneous case obtained approximately a month after clinical signs had healed. The virus employed was derived from the 8th, 10th, and 15th serial

rabbit passage of the Xy171 strain (2). A total of 28 pox immune serum-pox virus mixtures were tested on 6 rabbits.

The results showed that with two partial exceptions undiluted pox immune serum completely neutralized diluted and full strength pox virus, whereas normal serum possessed no neutralizing properties whatever. The findings on 2 rabbits are summarized in Tables I and II.

The partial exceptions occurred in 2 animals injected with the same serum-virus mixtures and comprised modified positive reactions to the mixtures containing full strength virus and the dilutions of 1:10 and 1:100. The protocol of 1 animal is given in Table II.

The lesions which did not develop before the 4th day were irregular somewhat nodular areas of cutaneous thickening with moderate congestion. There was little or no hemorrhage and no edema or necrosis, and within 2 days regression had begun. The sluggish non-progressive character of these lesions contrasted sharply with the actively progressing lesions produced by pox virus mixed with normal serum which began within 24 to 48 hours after injection and in which hemorrhage, edema, and necrosis were conspicuous even in virus dilutions of 1:10,000 and 1:100,000. This modified reaction, however, was not seen in another experiment in which the same specimen of serum completely neutralized all dilutions of pox virus.

The results of the experiments in which dilutions of pox immune serum as well as dilutions of virus were used will be described later in connection with the observations on the neutralizing action of vaccine immune serum.

Virus III Immune Serum-Pox Virus Tests.—Virus III immune serum did not exhibit any neutralizing properties against pox virus in the one experiment in which this combination was tested.

The serum was obtained 25 days after inoculation and in full strength combinations it completely neutralized the homologous virus. Pox virus in dilutions of 1:1 to 1:1,000 with full strength serum was employed (Table I). A characteristic cutaneous lesion was obtained with each mixture. They were entirely comparable to those resulting from the injection of normal serum-pox virus mixtures as regards time of development, appearance, and course of the lesions (Table I).

Since these results were in complete agreement, first, with those obtained in the crossed inoculation experiments with virus III and pox recovered rabbits in which the animals were found to be susceptible

TABLE I

Results of Serum Neutralization Tests with Rabbit Pox, Vaccinia, and Virus III Immune Sera and Rabbit Pox Virus. 0.2 Cc. Injected Intradermally

Immune sera	Rabbit pox virus	Cutaneous reaction. Days after injection						
		1	2	3	4	5	6	7
Pox	+ 1:1	±	±	−	−	−	−	−
	+ 1:10	±	+	−	−	−	−	−
	+ 1:100	−	−	−	−	−	−	−
	+ 1:1,000	−	−	−	−	−	−	−
Vaccinia	+ 1:1	±	++	++	+++	+++	+++	+++
	+ 1:10	±	+	++	++	+++	+++	+++
	+ 1:100	−	−	−	+	++	++	++
	+ 1:1,000	−	−	−	−	−	−	+
Virus III	+ 1:1	++	+++	++++	++++	++++	+++++	+++++
	+ 1:10	+	++	+++	+++	++++	++++	++++
	+ 1:100	−	+	++	++	+++	+++	+++
	+ 1:1,000	−	+	+	+	++	+++	+++
Normal	+ 1:1	+++	++++	++++	++++	++++	+++++	+++++
	+ 1:10	+++	+++	+++	++++	++++	++++	++++
	+ 1:100	+	++	+++	+++	+++	++++	++++
	+ 1:1,000	+	++	++	++	++	+++	+++

to inoculation with the heterologous virus, and second, with the finding that virus III immune rabbits contracted pox after room exposure (1), other serum neutralizing tests were not carried out.

Vaccine Immune Serum-Pox Virus Tests.—Vaccine immune serum was found to possess partial neutralizing properties against pox virus.

Both culture (dermo) vaccine and neurovaccine immune sera were used. The first experiments were carried out with sera of 2 rabbits inoculated with culture tissue-virus 17 and 24 days before blood was withdrawn. The serum-virus mixtures were similar to the pox immune serum-pox mixtures previously described, that is, virus dilutions up to 1:100,000 combined with full strength serum, and the injections numbering 28 were made on the same rabbits. The protocols of 2 animals are given in Tables I and II.

The character of the action of vaccine immune serum on pox virus can best be appreciated by recalling first the complete neutralizing properties of pox immune serum against pox virus described above, and second the high potency of the virus itself (Tables I and II).

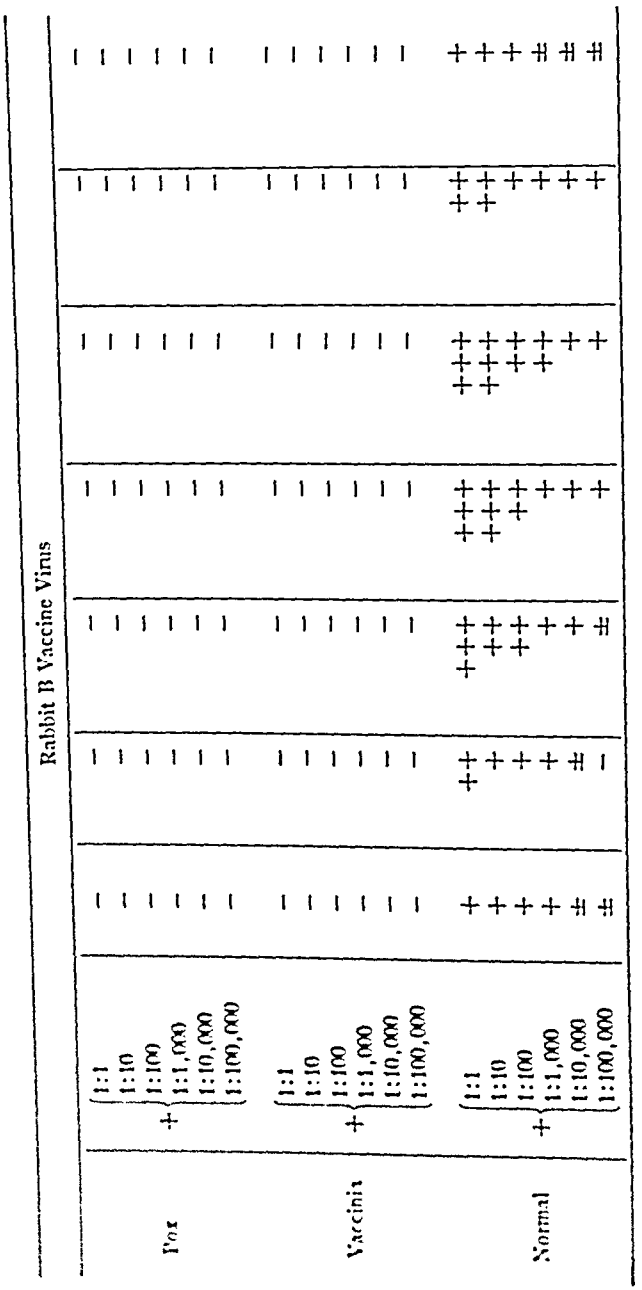
With normal serum-pox virus mixtures, a positive reaction with virus dilutions of 1:1 to 1:10,000 developed on the 1st and 2nd days and in the dilutions of 1:100,000 on the 3rd day. The cutaneous lesions increased very rapidly and soon comprised large swollen areas in which edema, congestion, hemorrhage, and necrosis were conspicuous features. It should be noted that as late as the 7th day little or no regression had occurred.

The cutaneous reactions resulting from injections of culture immune serum-pox virus mixtures developed later and were much less pronounced than those of the normal serum-pox virus mixtures, a finding which indicates a definite neutralizing action (Tables I and II). The effect was especially evident in the higher virus dilutions. For example, on the 4th day, as is shown in the protocol in Table II, the lesion of the 1:1,000 virus dilution with normal serum was pronounced (++++) while with vaccine immune serum the reaction was positive for the first time (+). The vaccine immune serum-pox virus lesions were smaller, shallower, and less destructive, and there was much less edema and necrosis. But it was clearly evident that in comparison with the marked neutralizing effect of pox immune serum against pox virus, vaccine immune serum was very much less potent. On the other hand, pox immune serum was just as effective in neutralizing

TABLE II

Results of Serum Neutralization Tests with Rabbit Pox and Vaccinia Immune Sera and Rabbit Pox and Vaccinia Viruses. 0.2 Cc. Injected Intradermally

Immune sera	Virus	Cutaneous reaction. Days after injection						
		1	2	3	4	5	6	7
Rabbit A Pox Virus								
Pox	+ 1:1	±	±	±	+	±	−	−
	+ 1:10	−	−	−	+	+	+	±
	+ 1:100	−	−	−	−	±	+	±
	+ 1:1,000	−	−	−	−	−	−	−
	+ 1:10,000	−	−	−	−	−	−	−
	+ 1:100,000	−	−	−	−	−	−	−
Vaccinia	+ 1:1	±	+++	+++	+++	++++	++++	Confluent
	+ 1:10	−	++	++	++	+++	+++	
	+ 1:100	−	+	+	+	++	++	++
	+ 1:1,000	−	−	±	+	++	+	+
	+ 1:10,000	−	−	±	−	+	+	±
	+ 1:100,000	−	−	−	−	−	−	−
Normal	+ 1:1	±	++++	+++++	+++++	Confluent	++++++	Confluent
	+ 1:10	±	++++	++++	+++++		+++++	
	+ 1:100	−	±	+++	+++++	+++++	+++++	
	+ 1:1,000	−	++	++	++++	++++	++++	++++
	+ 1:10,000	−	+	++	+++	+++	+++	+++
	+ 1:100,000	−	−	+	+	+	++	++


Rabbit B Vaccine Virus
Pox
Vaccinia
Normal
+ 1:1
1:10
1:100
1:1,000
1:10,000
1:100,000

culture vaccine virus as was vaccine immune serum (Table II, rabbit B).

In this connection the confirmatory results of another experiment are of interest. Neutralization tests were made with two specimens of serum from a rabbit, the first of which, serum A, was obtained 27 days after inoculation with culture vaccine virus and the second, serum B, which was obtained 14 days after the vaccinated rabbit had been inoculated with pox virus. The tests made on 2 rabbits comprised mixtures of serum A and serum B with pox virus and similar combinations with vaccine virus. Equal parts of full strength serum with 3 dilutions of virus, 1:1, 1:10, and 1:100 respectively were used. All the control tests comprising normal serum and virus dilutions of 1:10 and 1:100 were positive. In the case of the 12 mixtures containing vaccine virus, 6 with serum A and 6 with serum B, no reactions were observed, that is, complete neutralization of virus had been accomplished. In the case of the 6 mixtures containing pox virus and serum A (vaccine immune serum), well marked lesions developed from the 1:1 and 1:10 virus dilutions but not from the 1:100 dilutions, a result which is in accord with those previously described, namely, that vaccine immune serum has partial neutralizing properties against pox virus. On the other hand, the injections of the 6 mixtures of pox virus and serum B (vaccine and pox immune serum) gave negative results in all instances.

Of particular interest was the peculiar appearance and course of the cutaneous lesions produced by the vaccine immune serum-pox virus mixtures as is shown by the photographs in Figs. 1 to 4 taken on the 3rd, 4th, 5th, and 9th days respectively. The first visible reaction was observed 2 to 3 days after injection and comprised large circular pinkish yellow areas with rather ill defined margins which corresponded to slight but definite superficial cutaneous swellings (Fig. 1). Within 24 to 48 hours the swelling had increased markedly in depth, especially at the periphery, so that the lesions had a crater-like appearance. There was little if any increase in the diameter of the involved area. Particularly striking was the nodular character of the lesions which was most marked at the periphery (Figs. 2 and 3). The overlying skin became congested and some hemorrhage developed together with small punctate necroses. These features were also more conspicuous around the circumference than in the depressed central portion. Edema was not conspicuous, whereas in the case of intradermal injections of pox virus it was usually very pronounced and extensive. During the next 2 or 3 days the lesion usually became larger and more uniform in depth, thus losing something of its crater-

like appearance, but the areas of necrosis tended to preserve their peripheral distribution (Figs. 3 and 4). By the end of a week regression had begun and healing proceeded more rapidly than with the usual pox lesions, probably because tissue destruction was less extensive.

In some instances the lesions induced by vaccine immune serum-pox virus mixtures containing full strength virus failed to develop beyond the initial stage of swelling and congestion (Figs. 1 and 2) and underwent rapid regression and healing (Figs. 3 and 4). It might be thought that this result was due to specific antibodies contained in the tissue emulsion of pox virus which conditions of dilution in the other mixtures rendered incapable of effect. A similar finding was observed on two occasions with pox immune serum as has been previously described, but none was observed in the case of mixtures of full strength pox virus with normal or with virus III immune sera.

The experiments with neurovaccine[1] comprised a series of tests in which were compared the neutralizing properties of pox immune, culture vaccine immune, and neurovaccine immune sera against the three viruses.

Each virus was prepared from pooled testicular tissue from 3 rabbits with an acute orchitis. Pooled immune serum was used, each being composed of equal amounts of six specimens derived from different rabbits. The intervals between inoculation and bleeding varied from 17 to 72 days. Each test was carried out in duplicate by the intradermal injection of 0.2 cc. of each inoculum on 2 rabbits. Mixtures containing pox virus were injected on the right side of the body, those containing culture vaccine virus on the upper left, and those containing neurovaccine on the lower left side respectively.

The strength or titer of the three viruses was first determined by intradermal injections of dilutions ranging from 1:100 to 1:1,000,000.

[1] The experiments with neurovaccine were restricted by the available space which could be satisfactorily quarantined. Three individuals were allowed access to the two rooms set aside for this work, and these persons did not go to other animal rooms or laboratories while the experiments were in progress. A strict routine of hand disinfection and change of laboratory coats was maintained. At the conclusion of the experiments, all remaining stock was killed and the rooms were thoroughly disinfected with lysol. As far as is known, no spread of neurovaccinia occurred.

The results which are summarized in the following tabulation showed that positive reactions were obtained by 1:1,000,000 dilutions of both pox and neurovaccine viruses and by a 1:100,000 dilution of culture vaccine virus. The highest dilutions which produced comparable cutaneous reactions of fair size, however, were: pox virus 1:100,000, neurovaccine virus 1:10,000, and culture vaccine virus 1:1,000.

Preliminary Test on the Potency of the Viruses

Virus dilution		Rabbit A	Rabbit B
Rabbit pox	0.01	+++++	++++
	0.001	++++	+++
	0.0001	+++	++
	0.00001	++	++
	0.000001	++	+
Neurovaccine	0.01	++++	+++
	0.001	+++	++
	0.0001	++	+
	0.00001	++	+
	0.000001	+	±
Culture vaccine	0.01	+++	++
	0.001	++	++
	0.0001	+	+
	0.00001	±	±
	0.000001	0	0

The neutralizing potency of each immune serum against its homologous virus was next tested by intradermal injection of serum-virus mixtures in which varying dilutions of serum were mixed with a constant dilution of virus. 8 dilutions of serum ranging from 1:10 to 1:5,000,000 were employed. The virus dilutions used were selected on the basis of the previous findings, that is, pox 1:100,000, neurovaccine 1:10,000, and culture vaccine 1:1,000.

The results, the relevant portions of which are summarized in the following tabulation, showed that the highest dilution of pox immune serum which neutralized pox virus (1:100,000) was 1:100; that of neurovaccine immune serum which neutralized neurovaccine virus (1:10,000) was 1:10; and that of culture vaccine immune serum which neutralized culture vaccine virus (1:1,000) was 1:10.

Preliminary Tests on the Potency of the Immune Sera

Virus dilution		Serum dilution	Rabbit A	Rabbit B
Rabbit pox	1:100,000 + pox immune	1:10	0	0
		1:100	0	0
		1:1,000	+	++
		1:10,000	++++	+++
Neurovaccine	1:10,000 + neurovaccine immune	1:10	0	0
		1:100	+	+++
		1:1,000	+++	++
		1:10,000	++	+++
Culture vaccine	1:1,000 + culture vaccine immune	1:10	0	0
		1:100	+	+
		1:1,000	+	+++
		1:10,000	+	+++
Rabbit pox	1:100,000 + normal	1:1	++	+++
Neurovaccine	1:10,000 + "	1:1	+++	+++
Culture vaccine	1:1,000 + "	1:1	+++	+++

With this information on the titer of the three viruses and the potency of each immune serum against its homologous virus, 2 experiments were carried out in which each serum was tested for its neutralizing properties against the heterologous viruses. The serum-virus mixtures in one experiment comprised a constant dilution of virus with varying dilutions of serum and in the other, varying dilutions of virus with a constant dilution of serum. The results are summarized in Tables III and IV.

In the first experiment pox virus in a dilution of 1:100,000 was used (Table III). This dilution was neutralized in both test animals by pox immune serum diluted 1:100 and in one animal by a 1:1,000 dilution. It was also neutralized by a 1:10 dilution of neurovaccine immune serum and in one animal by a 1:100 dilution while with culture vaccine immune serum the neutralization level was 1:10 in both rabbits.

The dilution of neurovaccine virus employed, 1:10,000, was ten times as concentrated as the dilution of pox virus used. The upper level of neutralization with both neurovaccine immune serum and culture serum was the dilution of 1:10, but in the case of pox immune serum it was 1:100 in both animals and 1:1,000 in one of them.

With culture vaccine virus only the results on one rabbit are available. The

TABLE III

Results of Crossed Immune Serum Neutralization Tests. Constant Dilutions of Virus and Varying Dilutions of Serum

Virus	Serum dilution	Rabbit pox immune serum		Neurovaccine immune serum		Culture vaccine immune serum		Normal serum	
		A	B	A	B	A	B	A	B
Rabbit pox 1:100,000	+ 1:1	−	−	−	−	−	−	++	++
	+ 1:10	−	−	−	−	−	−		
	+ 1:100	−	−	+	−	++	+		
	+ 1:1,000	−	+	++	+	+++	++		
	+ 1:10,000	++	++	+++	++	+++	++		
	+ 1:100,000	+++	++	+++	++	+++	++		
		C	D	C	D	C	D	C	D
Neurovaccine 1:10,000	+ 1:1	−	−	−	−	−	−	++	+
	+ 1:10	−	−	−	−	−	−		
	+ 1:100	−	−	+	+	++	+		
	+ 1:1,000	−	++	+	+	+	++		
	+ 1:10,000	+	++	+	++	+	++		
	+ 1:100,000	++	+	++	++	+	+		
		E		E		E		E	
Culture vaccine 1:1,000	+ 1:1	−		−		−		++	
	+ 1:10	−		−		−			
	+ 1:100	−		−		+			
	+ 1:1,000	−		−		++			
	+ 1:10,000	++		++		++			
	+ 1:100,000	++		++		++			

0.2 cc. of serum-virus mixtures injected intradermally. Pox virus mixtures injected in rabbits A and B; neurovaccine virus mixtures injected in rabbits C and D; culture vaccine mixtures injected in rabbits E and F; rabbit F discarded because of poor physical condition.

dilution of virus employed was 1:1,000, or ten times that of neurovaccine and 100 times that of pox virus. Neutralization with the homologous culture vaccine immune serum occurred in the 1:10 but not in the higher dilutions, while with both neurovaccine and pox immune sera, neutralization included the 1:1,000 dilutions.

These results indicate a degree of similarity or inter-relationship between the three immune sera. As was to be expected, the neutralizing properties of each serum for its homologous virus were demonstrated, thus confirming the previous findings obtained in the preliminary tests; but in addition it was found that each serum also possessed some measure of neutralizing action against the two heterologous viruses. Pox immune serum had a potent neutralization action against pox virus, but the 2 lower dilutions of both neurovaccine and culture vaccine immune sera also neutralized pox virus. Pox immune serum was more potent against neurovaccine virus than either neurovaccine or culture vaccine immune serum; and it was more effective against culture vaccine virus than culture vaccine immune serum. Culture vaccine and neurovaccine immune sera gave almost identical results with respect to the dilutions at which they neutralized pox and neurovaccine viruses; in both cases the sera were not as potent as pox immune serum. On the other hand, the tests with culture vaccine virus showed that it was neutralized by the same dilutions of neurovaccine immune serum and pox immune serum, and furthermore that both these sera were more potent than culture vaccine immune serum. If only the results with pox and neurovaccine viruses are considered, the similar findings for neurovaccine and culture immune sera speak for their identity, while the differences between these findings and those for pox immune serum would indicate that the latter serum is not identical with the other two. The results with culture vaccine virus, however, suggest the identity of pox and neurovaccine immune sera, and a difference between them and the homologous culture vaccine immune serum.

The second neutralization experiment comprised constant dilution of each immune serum in combination with varying dilutions of the three viruses (Table IV). 5 virus dilutions in multiples of ten were employed, beginning with 1:1,000 in the case of pox virus and 1:1 in the case of both culture vaccine and neurovaccine virus. The particular dilution of serum selected was the highest which neu-

tralized a particular amount of homologous virus as determined by the results of the preliminary tests previously described on the strength or potency of the sera, that is, pox serum 1:100, neurovaccine serum 1:10, and culture vaccine serum 1:10.

TABLE IV

Results of Crossed Immune Serum Neutralization Tests. Constant Dilutions of Serum and Varying Dilutions of Virus

Immune serum	Virus dilution	Rabbit pox virus		Neurovaccine virus		Culture vaccine virus	
		A	B	A	B	A	B
Rabbit pox 1:100	1:1			++	++	−	−
	1:10			+	++	−	−
	1:100			−	++	−	−
	1:1,000	+	+++	−	±	−	−
	1:10,000	−	++	−	−	−	−
	1:100,000	−	−				
	1:1,000,000	−	−				
	1:10,000,000	−	−				
		C	D	C	D	C	D
Neurovaccine 1:10	1:1			++	+	−	−
	1:10			+	+	−	−
	1:100			±	−	−	−
	1:1,000	+++	++	−	−	−	−
	1:10,000	+	+	−	−	−	−
	1:100,000	+	−				
	1:1,000,000	−	−				
	1:10,000,000	−	−				
		E	F	E	F	E	F
Culture vaccine 1:10	1:1			++	+	++	+
	1:10			±	+	+	+
	1:100			+	±	−	−
	1:1,000	++	+	−	−	−	−
	1:10,000	++	+	−	−	−	−
	1:100,000	−	−				
	1:1,000,000	−	−				
	1:10,000,000	−	−				

0.2 cc. of serum-virus mixtures injected intradermally. Pox serum mixtures injected in rabbits A and B; neurovaccine serum mixtures injected in rabbits C and D; culture vaccine serum mixtures injected in rabbits E and F.

The results of this experiment (Table IV) showed that pox immune serum (1:100) neutralized the 3 highest dilutions of pox virus,

1:10,000,000 to 1:100,000 and one of the two 1:10,000 dilutions. It neutralized neurovaccine, however, in the 1:10,000 and 1:1,000 dilutions of virus and in one of the 1:100 dilutions. All the dilutions of culture vaccine virus were neutralized.

With neurovaccine immune serum (1:10) neutralization of neurovaccine virus occurred in the virus dilutions of 1:10,000 and 1:1,000 (Table IV). All the dilutions of culture vaccine virus were neutralized. On the other hand, neurovaccine immune serum did not neutralize pox virus in its 1:10,000 dilutions nor in one of the 1:100,000 dilutions. It should be pointed out, however, that in the previous experiment both the 1:100,000 dilutions of pox virus were neutralized by the 1:10 dilution of neurovaccine immune serum (Table III).

The tests with culture vaccine immune serum (1:10) showed neutralization of culture vaccine virus in the 3 higher dilutions beginning with 1:100. The serum neutralized the pox virus dilutions of 1:100,000 and above but not those of 1:1,000 and 1:10,000. The reactions with neurovaccine virus were somewhat irregular. Virus dilutions of 1:1,000 and 1:10,000 were neutralized; in one each of the 1:10 and 1:100 dilutions neutralization was not obtained, but the results of the duplicate dilutions were equivocal.

From these observations it appears that pox immune serum in dilutions of 1:100 was more efficacious in its neutralizing properties against pox virus than either neurovaccine or culture vaccine immune serum in dilutions of 1:10; furthermore, that its effect against neurovaccine virus was almost as marked as that of neurovaccine immune serum despite the tenfold difference in concentration; and finally, that it was more effective against culture vaccine virus than ten times the concentration of culture vaccine immune serum. In addition, the neutralizing action of pox immune serum (1:100) was more powerful against both neurovaccine and culture vaccine viruses than against pox virus. Neurovaccine immune serum was more efficacious against neurovaccine virus and culture vaccine virus than against pox virus. And culture vaccine immune serum was also more potent against culture vaccine and neurovaccine virus than against pox virus. Pox immune serum in the dilution used, 1:100, neutralized the 1:10,000 dilutions of neurovaccine virus, one and probably both the dilutions of 1:1,000 and one of the 1:100 dilutions. On the other hand, neuro-

vaccine immune serum in dilutions of 1:10 did not neutralize pox virus in dilutions of 1:1,000 and 1:10,000 nor in 1 of 4 tests with a 1:100,000 dilution (Tables III and IV). Pox immune serum diluted 1:100 neutralized all dilutions of culture vaccine including full strength suspensions. Culture vaccine immune serum diluted 1:10 did not neutralize pox virus diluted 1:1,000 and 1:10,000 although it neutralized culture vaccine virus at 1:100 and neurovaccine virus at 1:1,000.

Other comparisons could be made, but enough has been said to indicate the essential features of the findings. Bearing in mind that both neurovaccine immune and culture vaccine immune sera were used in dilutions ten times as concentrated as those of pox immune serum, the results may be summarized as follows: First, the neutralizing properties of pox immune serum against pox virus, neurovaccine virus, and culture vaccine virus were more powerful than those of neurovaccine immune serum or culture vaccine immune serum. And second, with respect to the strength or virulence of the viruses as shown by the cutaneous reactions, the titer of pox virus was higher than that of either neurovaccine virus or culture vaccine virus.

While each immune serum displayed some neutralizing action against the two heterologous viruses, the range of this action as determined by the results of parallel dilutions did not coincide with that shown against the homologous virus. The sera possessed in variable degree common neutralizing properties but complete identity between them could not be demonstrated. It follows, therefore, that the viruses with which the sera were produced possessed some common antigenic relationship but that they were not completely identical one with another.

SUMMARY AND CONCLUSIONS

The results of serum neutralizing tests with the viruses of pox, vaccinia, and virus III disease herewith reported generally agree and supplement the results of reinoculation experiments on immune rabbits reported in the previous paper (1).

The finding that pox virus is neutralized by pox immune serum indicates that the refractory state of recovered pox rabbits to reinoculation with pox virus and the failure of recovered pox rabbits to con-

tract a second pox infection after adequate exposure is to be explained upon the basis of the development of an active immunity.

The failure of virus III immune serum to neutralize pox virus is in agreement with the previous conclusion drawn from the positive results of reinoculation and exposure experiments that there is no specific relationship between pox virus and virus III.

Rabbits which had recovered from a pox infection were completely refractory to inoculation with dermo- (culture) vaccine, while rabbits which had recovered from vaccinia were partially refractory to inoculation with pox virus. Vaccine immune adult rabbits did not show any clinical evidence of pox upon exposure to florid cases, but young recently weaned vaccine immunes developed definite although comparatively mild pox infections. The serum neutralization tests showed that pox immune serum neutralized vaccine virus although the action was not complete as shown by the positive results obtained with high dilutions of serum; vaccine immune serum possessed some but comparatively slight neutralizing properties against pox virus. The results of the crossed inoculation and serum neutralizing experiments with pox and neurovaccine viruses resembled those obtained with pox and dermo- (culture) vaccine but the differences were less pronounced. The differences in virus neutralizing ability on the part of the three immune sera paralleled the differences in virus potency as indicated by the character of the local lesions at the site of injection and by the general character of the clinical manifestations of the infection. The potency or virulence of pox virus was much greater than that of neurovaccine and vastly greater than that of dermo- (culture) vaccine.

The complete identity of pox and vaccine virus could not be established, but a definite relationship between them was demonstrated and this was shown to have an immunological basis. From a practical standpoint vaccination with vaccine virus as a prophylactic measure against rabbit pox was clearly indicated.

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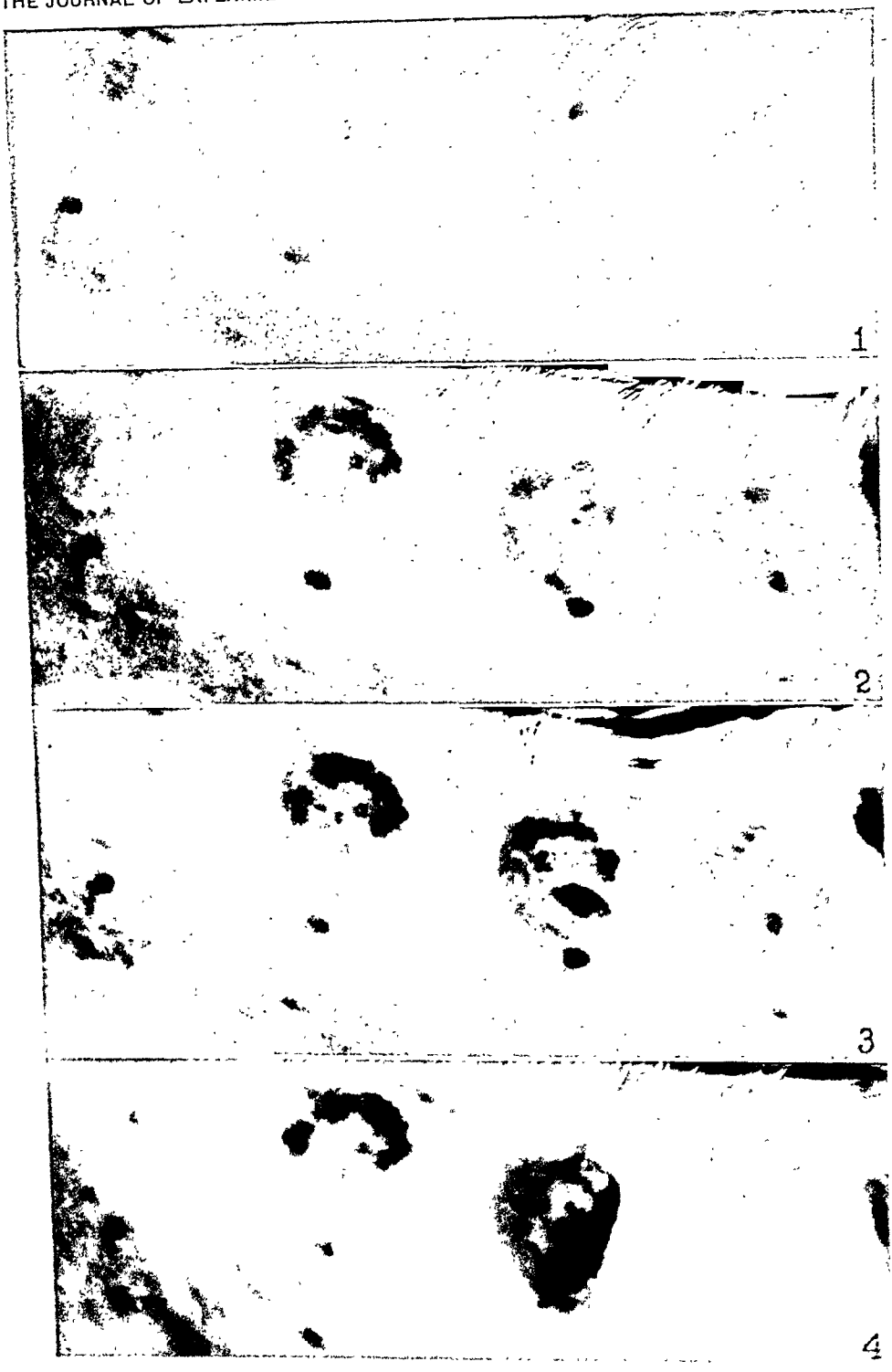
EXPLANATION OF PLATE 29

FIGS. 1 through 4. 3, 4, 5, and 9 days respectively after injection. Evolution of the modified cutaneous lesions of rabbit pox virus which developed after intradermal injection of a rabbit with mixtures of full strength culture vaccine immune serum and pox virus diluted 1:1, 1:10, 1:100, and 1:1,000. The complete neutralization of pox virus in the same dilutions by pox immune serum is shown by the negative cutaneous results in the injected areas just below the lower row of black dots.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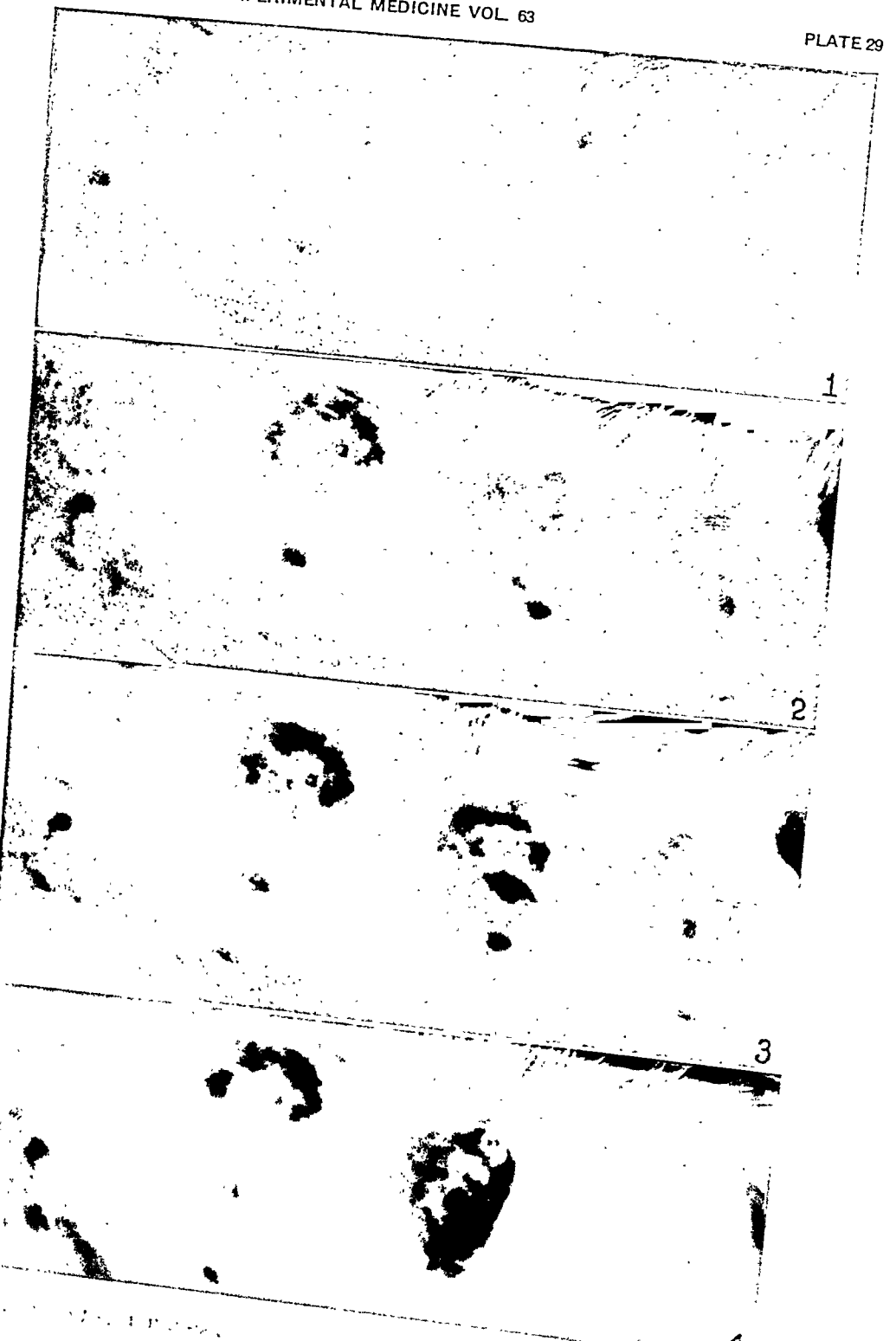
EXPLANATION OF PLATE 29

FIGS. 1 through 4. 3, 4, 5, and 9 days respectively after injection. Evolution of the modified cutaneous lesions of rabbit pox virus which developed after intradermal injection of a rabbit with mixtures of full strength culture vaccine immune serum and pox virus diluted 1:1, 1:10, 1:100, and 1:1,000. The complete neutralization of pox virus in the same dilutions by pox immune serum is shown by the negative cutaneous results in the injected areas just below the lower row of black dots.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2
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arithmetic. There was photophobia and some weakness of convergence; otherwise the cranial nerves were normal, and there were no changes in the optic fundi. There was definite but not marked nuchal rigidity, but Kernig's sign was absent. Motor power, sensation, and position sense appeared normal. The biceps reflexes were moderately active; the triceps reflex was obtained on the right but not on the left side. Both knee jerks and ankle jerks were moderately active. Plantar, abdominal, and cremasteric reflexes were present and normal.

Course.—A lumbar puncture performed on admission revealed a slightly turbid spinal fluid that was under increased pressure and contained 1700 cells per c.mm., mainly lymphocytes (Chart 1). During the 24 hours after admission the knee jerks disappeared and the plantar reflexes became equivocally extensor in type. Within 4 days, however, the knee jerks returned, the left more fully than the right, and the plantar reflexes were only very transitorily abnormal. During the first 4 days in hospital the lumbar puncture was repeated twice, following which the patient's general condition improved rapidly. At the end of this time his temperature had fallen to the normal level (Chart 1) and he had no definite symptoms, although his neck was still stiff on examination. During the first 16 days in the hospital the patient's bowels were only moved with the aid of enemata, but at the end of that time he began to have spontaneous bowel movements, and thereafter, the intestinal motility remained normal. By the 26th of January, 43 days after onset, the cells in the spinal fluid had decreased in number until there were only 25 per c.mm. At that time the patient was allowed to get up. On Feb. 6, the spinal fluid showed only 8 cells per c.mm. and a heavy trace of globulin. On Feb. 7, 56 days after onset, since a complete examination showed a normal central nervous system, the patient was discharged from the hospital. He has been back at work for 8 months and a recent neurological examination revealed no abnormalities.

Spinal Fluid.—Dec. 22, 1934: slightly turbid, under increased pressure; cells, predominantly lymphocytes, 1700 per c.mm.; no organisms could be found in stained preparations or in cultures; protein 100 mg. per 100 cc.; sugar 39 mg. per 100 cc. *Dec. 23:* turbid, under pressure of 115 mm. of water; 1200 cells per c.mm., 97 per cent being lymphocytes. *Dec. 24:* clear, under pressure of 150–170 mm. of water; 1100 cells per c.mm., 95 per cent of which were lymphocytes; Nonne-Apelt test was positive; a portion of the fluid was injected into a guinea pig to test for the presence of tubercle bacilli, and the animal was found dead 16 days later with pulmonary congestion but no evidence of tuberculosis. *Dec. 31:* slightly turbid; 740 cells per c.mm.; protein 100 mg. per 100 cc.; spinal fluid sugar 43 mg. per 100 cc., blood sugar 83 mg. per 100 cc. *Jan. 7, 1935:* clear; 100 cells per c.mm. *Jan. 19:* pink, 35 cells per c.mm. *Jan. 26:* clear, with a faint yellowish tinge; 25 cells per c.mm.; protein 70 mg. per 100 cc.; sugar 68 mg. per 100 cc.; colloidal gold test yielded 5554342100, but the fluid was xanthrochromic; Wassermann test on 0.1 cc. of fluid was negative. *Feb. 6:* clear; 8 cells per c.mm.; an excess of globulin was present.

Examinations for tubercle bacilli in the sediment from 3 samples of spinal

the day of admission, 8 days after the first symptoms, and 3 days after the onset of the severe headache, he became drowsy, and, when examined by a physician, was found to have a stiff neck, a Kernig sign, and a temperature of 102°F. A tentative diagnosis of post-influenzal encephalitis was made, and he was sent to the New York Hospital.

Physical Examination.—Upon admission the patient's rectal temperature was 104°F., pulse 110, respirations 34, and blood pressure 100/80 mm. Hg (Chart 1).

Spinal fluid	Sugar mg. per 100 cc.	39				
	Protein • • • •	100				
	Globulin		+	+		
	Culture		−			
	Mouse inoculation		+			
	Red blood cells	−	+	−		
	Leucocytes per c.mm.	1700	1200	1100		
	Per cent mononuclear cells	Mainly	97	95		
Blood	Culture			−		
	Mouse inoculation		−			
	Leucocytes per c.mm.	8800	5700			
	Per cent granulocytes	55	57			
	Temperature (°F.) 104 103 102 101 100 99 98 / Pulse 130 120 110 100 90 80 70 — Temp. Pulse					
	December, 1934	22	23	24	25	26
	Day of disease	8	9	10	11	12

CHART 1. Studies on Case I (W.E.) during first 5 days in hospital.

He was semistuporous, but could be aroused sufficiently to answer questions; he moaned at intervals and kept his eyes tightly shut; he tended to lie on his right side with thighs flexed on the abdomen, a position the legs still maintained when he was turned on his back. No lead line was present; there was no cutaneous evidence of herpes, and, apart from dry, crusted lips, the rest of the physical examination, except for the nervous system, was negative.

Neurological Examination.—Mentally he was coherent in spite of drowsiness; he was somewhat confused as to time relationships but could handle simple

arithmetic. There was photophobia and some weakness of convergence; otherwise the cranial nerves were normal, and there were no changes in the optic fundi. There was definite but not marked nuchal rigidity, but Kernig's sign was absent. Motor power, sensation, and position sense appeared normal. The biceps reflexes were moderately active; the triceps reflex was obtained on the right but not on the left side. Both knee jerks and ankle jerks were moderately active. Plantar, abdominal, and cremasteric reflexes were present and normal.

Course.—A lumbar puncture performed on admission revealed a slightly turbid spinal fluid that was under increased pressure and contained 1700 cells per c.mm., mainly lymphocytes (Chart 1). During the 24 hours after admission the knee jerks disappeared and the plantar reflexes became equivocally extensor in type. Within 4 days, however, the knee jerks returned, the left more fully than the right, and the plantar reflexes were only very transitorily abnormal. During the first 4 days in hospital the lumbar puncture was repeated twice, following which the patient's general condition improved rapidly. At the end of this time his temperature had fallen to the normal level (Chart 1) and he had no definite symptoms, although his neck was still stiff on examination. During the first 16 days in the hospital the patient's bowels were only moved with the aid of enemata, but at the end of that time he began to have spontaneous bowel movements, and thereafter, the intestinal motility remained normal. By the 26th of January, 43 days after onset, the cells in the spinal fluid had decreased in number until there were only 25 per c.mm. At that time the patient was allowed to get up. On Feb. 6, the spinal fluid showed only 8 cells per c.mm. and a heavy trace of globulin. On Feb. 7, 56 days after onset, since a complete examination showed a normal central nervous system, the patient was discharged from the hospital. He has been back at work for 8 months and a recent neurological examination revealed no abnormalities.

Spinal Fluid.—Dec. 22, 1934: slightly turbid, under increased pressure; cells, predominantly lymphocytes, 1700 per c.mm.; no organisms could be found in stained preparations or in cultures; protein 100 mg. per 100 cc.; sugar 39 mg. per 100 cc. *Dec. 23:* turbid, under pressure of 115 mm. of water; 1200 cells per c.mm., 97 per cent being lymphocytes. *Dec. 24:* clear, under pressure of 150–170 mm. of water; 1100 cells per c.mm., 95 per cent of which were lymphocytes; Nonne-Apelt test was positive; a portion of the fluid was injected into a guinea pig to test for the presence of tubercle bacilli, and the animal was found dead 16 days later with pulmonary congestion but no evidence of tuberculosis. *Dec. 31:* slightly turbid; 740 cells per c.mm.; protein 100 mg. per 100 cc.; spinal fluid sugar 43 mg. per 100 cc., blood sugar 83 mg. per 100 cc. *Jan. 7, 1935:* clear; 100 cells per c.mm. *Jan. 19:* pink, 35 cells per c.mm. *Jan. 26:* clear, with a faint yellowish tinge; 25 cells per c.mm.; protein 70 mg. per 100 cc.; sugar 68 mg. per 100 cc.; colloidal gold test yielded 5554342100, but the fluid was xanthrochromic; Wassermann test on 0.1 cc. of fluid was negative. *Feb. 6:* clear; 8 cells per c.mm.; an excess of globulin was present.

Examinations for tubercle bacilli in the sediment from 3 samples of spinal

fluid were negative. There was never any demonstrable abnormality in the hydrodynamics of the spinal fluid.

Blood.—Dec. 22, 1934: hemoglobin 100 per cent; red blood cells 5,300,000; white blood cells 8800; differential count yielded 40 per cent adult and 15 per cent immature polymorphonuclear neutrophils, 35 per cent lymphocytes, 9 per cent monocytes, and 1 per cent eosinophils. *Dec. 23:* white blood cells, 5700; differential count yielded 45 per cent adult and 12 per cent immature polymorphonuclear neutrophils, 44 per cent lymphocytes, 8 per cent monocytes, and 1 per cent eosinophils; Kline test was negative; urea nitrogen 11 mg. per 100 cc.; blood sugar 71 mg. per 100 cc. *Dec. 24:* blood for culture was taken and remained sterile for 5 days at 37°C. *Jan. 29, 1935:* convalescent serum failed to neutralize the virus of the St. Louis type of encephalitis.[3]

Urine.—Normal.

Search for Etiological Agent in Case I

The clinical picture presented by the patient suggested that he was suffering from a meningitis, while the type of cellular reaction evident in the spinal fluid,—in which ordinary bacteria were not demonstrated,—led us to believe that the etiological agent might be a virus. In order to test this idea blood and spinal fluid were collected from the patient on the day after admission for inoculation into animals.

Spinal Fluid.—4 or 5 cc. of slightly blood-stained spinal fluid were obtained on Dec. 23, 1934, and used, within 1 to 2 hours after collection, for the inoculation of mice.[4] Each of 6 mice received 0.03 cc. of the spinal fluid intracerebrally and 0.5 cc. intraperitoneally. One of the animals on the 3rd day after inoculation was found to have a streptococcal infection of the brain and was discarded. Of the remaining 5 mice, 2 became sick on the 6th day after inoculation and were sacrificed for passage, 1 died on the 9th day and was discarded, and 2 became sick on the 9th day but recovered. The brains of the animals that were killed on the 6th day were removed aseptically. Aerobic and anaerobic cultures made from the brains in broth showed no growth after incubation for 48 hours at 37°C. At the end of this time, the brains, which in the interval had been kept at 5°C., were ground with Locke's solution into a 20 per cent emulsion which was used for the inoculation of 5 normal mice, 0.03 cc. and 0.25 cc. of the emulsion being given intracerebrally and intraperitoneally, respectively, into each of the animals. One mouse died as a result of the inoculations. Of the other 4, 2 died and 2 appeared sick on the 7th day after inoculation. The sick mice were sacrificed and their brains were removed. The brain from one of the mice that died was also removed.

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[3] Dr. L. T. Webster tested for the presence, in W.E.'s and R.E.S.'s blood, of neutralizing antibodies against the St. Louis type of encephalitic virus.

[4] Albino Swiss mice, usually 4–5 weeks old, were used.

All of the brains were found to be free from ordinary bacteria by means of aerobic and anaerobic cultures in broth. 0.03 cc. of a 20 per cent emulsion of these brains were inoculated into each of 5 normal mice by the intracerebral route alone. All 5 animals became ill on the 6th day after inoculation. Since that time the active agent has been passed serially through 15 sets of mice by means of intracerebral inoculations of emulsions, usually of 10 per cent strength, of mouse brains which have been shown to be free from ordinary bacteria.

Blood.—Blood drawn on Dec. 23 into a suitable amount of sterile heparin was used within 1 to 2 hours after collection for the inoculation of 6 mice, each animal receiving 0.03 cc. intracerebrally and 0.75 cc. intraperitoneally. The mice were observed closely for a month but showed no symptoms of ill health, and, when inoculated later with potent virus, were found to be susceptible.

Case II

R. E. S., Rockefeller Hospital No. 9373, white male, 33 years of age, was admitted to the Hospital of The Rockefeller Institute, Dec. 27, 1934. He was on the scientific staff of The Rockefeller Institute at Princeton, N. J., and had been investigating several virus diseases of animals, including pseudorabies. He had also been intimately exposed to mumps which his two children had contracted a fortnight previously. The patient, however, had a definite history of having had the disease himself in childhood. The present illness which at first was thought to be grippe started 12 days before admission with fever, headache, and generalized muscular pain. By the 5th day after onset the symptoms had decreased to the extent that he was able to return to his laboratory where he worked all day. That evening he suddenly developed a new and a very severe headache, which steadily became worse during the next 5 days, and which was unrelieved by aspirin. He remained in bed, but sleep was considerably disturbed by pain. He was able to take or retain very little nourishment because of nausea and vomiting; the vomiting was not projectile. Some photophobia and pain on moving the eyes were present during this time. He again ran a fever, the exact extent of which was not known, but on one occasion his oral temperature was 101.4°F. 5 days after the onset of his second headache the patient developed stiffness, pain, and soreness of the muscles of his back. He was then admitted to a hospital in Princeton with the diagnosis of influenza or brain tumor. 2 days later, 12 days after the onset of his illness and 7 days after the beginning of his second headache, he was transferred to the Hospital of The Rockefeller Institute with a tentative diagnosis of encephalitis.

Physical Examination.—Upon admission the patient's rectal temperature was 101.2°F., pulse 72, respirations 18, and blood pressure 112/78 mm. Hg. He showed evidences of a moderate degree of dehydration; the mucous membranes of the mouth were dry and desquamating, and there was a glossitis of the anterior 5th of the tongue. There was a blotchy injection of the soft palate, and the nasal mucous membranes were moderately hyperemic; there was a slight amount of

mucopurulent discharge in the nose the septum of which deviated to the left. Apart from the nervous system, no other abnormal physical signs were found.

Neurological Examination.—Mentally the patient was alert and cooperative. There was a slow hippus of both pupils, and a haziness in the outline of the temporal borders of both optic discs, accompanied by fulness of the veins, was noted. Otherwise the cranial nerves were normal. There was definite slight nuchal rigidity, but the presence of a Kernig's sign was questionable. Motor power and sense of position were normal, but a careful sensory examination revealed an area in the right lumbar region, supplied by the posterior branches of the 7th to 10th dorsal nerves, in which there was some diminution of sensation to pin prick. Definite tenderness to pressure over the erector spinae muscles was noted, and at times a mass contraction of these groups of muscles could be elicited by stroking the skin of the back. There was also tenderness along the costal margin and in the abdominal muscles on the right side. The deep reflexes throughout the body were moderately hyperactive, but could not be considered pathological. Babinski's sign was not obtained. The abdominal reflexes were at times readily elicited, but at other times were unobtainable in certain segments. Cremasteric reflexes were normal.

Course.—The spinal fluid, obtained on admission, was under normal pressure, was opalescent, and contained 720 mononuclear cells per c.mm. and an increased amount of globulin. Following lumbar puncture and the intravenous injection on two occasions of 50.0 cc. of a 50 per cent solution of glucose, the patient's headache diminished in severity and the nausea disappeared entirely. He was then able to enjoy and retain a normal diet, and his appetite rapidly returned to its usual excellence. The muscular tenderness continued for about 6 days gradually getting less, while the hypoesthesia over the back lasted for 3 weeks. During this time the patient occasionally complained of pain in a small area in each axilla, but these subjective phenomena were unassociated with abnormal physical findings. The temperature fell to normal within 48 hours after admission to the hospital; the pulse rate remained relatively slow, averaging between 60 and 70 per minute for the first 14 days, his normal rate being between 70 and 80. Some stiffness of the neck remained for 16 days. During the first 15 days in hospital, he experienced a good deal of abdominal distention, and his bowels did not move except with the aid of enemata, but after this time they moved spontaneously. Lumbar punctures were performed 4 times during the course of the illness, and on each occasion the amount of pleocytosis in the spinal fluid was less than that revealed by the preceding puncture. The patient was allowed to sit up and be out of bed 29 and 43 days, respectively, after the onset of illness. On Jan. 25, 2 days before he got up, the spinal fluid still contained 17 mononuclear cells per c.mm. and an increased amount of protein. On Feb. 3, 1935, 49 days after the onset of the illness, he was discharged at which time his physical examination was entirely negative except for some weakness induced by the long stay in bed. Following return to work he noticed some intention tremor of the right hand which lasted 3 months. On Sep. 20, 1935, the patient was free from symptoms and a neurological examination was negative.

Spinal Fluid.—Dec. 27, 1934: slightly opalescent and under a pressure of 60 mm. of water; Pandy positive; 720 cells per c.mm., of which 82 and 18 per cent were small and large mononuclear elements, respectively. No organisms were demonstrated in stained preparations or by means of cultures. A very fine pellicle formed on standing. The guinea pig inoculated with the fluid revealed no evidences of tuberculosis after being observed for 6 weeks. *Jan. 3, 1935:* clear, under normal pressure; Pandy positive; 185 cells per c.mm., of which 67 per cent were small and 33 per cent were large mononuclear elements; colloidal gold reaction was weakly positive, 1233321000. *Jan. 14:* clear, under normal pressure; Pandy positive; 55 cells per c.mm., of which 92 per cent were small mononuclear cells, 4 per cent large mononuclear elements, and 4 per cent polymorphonuclear cells. Colloidal gold reaction was 1221000000. *Jan. 25:* clear; Pandy positive; 17 cells per c.mm.; a few fresh red blood cells were present; colloidal gold reaction was 0122100000; Wassermann reaction was negative.

The hydrodynamics of the spinal fluid were always normal.

Blood.—Dec. 25, 1934 (Princeton Hospital): white blood cells 7100; differential count revealed 81 per cent polymorphonuclear neutrophils, 17 per cent lymphocytes, 2 per cent monocytes, and 2 per cent eosinophils. *Dec. 28:* hemoglobin (Sahli) 96 per cent; red blood cells 5,600,000 per c.mm.; white blood cells 9900 per c.mm.; differential count revealed 79 per cent polymorphonuclear neutrophils, 15 per cent lymphocytes, 4 per cent monocytes, 1 per cent eosinophils, and 1 per cent basophils; the blood culture remained sterile for 6 days at 37°C.; a reading of the Wassermann reaction was not possible because the serum was anticomplementary; Kahn test was negative. *Jan. 29, 1935*: convalescent serum failed to neutralize the virus of the St. Louis type of encephalitis.[3]

Urine.—Normal.

Search for Etiological Agent in Case II

Because the clinical picture presented by R.E.S. was similar to that described for W.E., cerebrospinal fluid and blood were also collected from him on admission for inoculation into animals.

Spinal Fluid.—Approximately 10.0 cc. of an opalescent spinal fluid were obtained from the patient on admission, Dec. 27, 1934, and were used within 2 hours after collection for the inoculation of mice[4] and rabbits.

Mice.—Each of 6 mice was inoculated intracerebrally with 0.03 cc. and intraperitoneally with 0.25 cc. of spinal fluid. While the animals were still under ether anesthesia, 0.02 cc. of the spinal fluid were dropped into the nostrils of each one. On the 8th day after inoculation 1 mouse was found dead; the other 5 appeared perfectly well and remained so. The brain of the mouse that died was removed aseptically, and aerobic and anaerobic cultures made from it in broth were found to be free from ordinary bacteria after incubation at 37°C. for 24 hours. The brain, which, in the meantime, had been kept at 5°C. was ground with Locke's solution into a 20 per cent emulsion of which 0.03 cc. and 0.12 to 0.25 cc., respec-

mucopurulent discharge in the nose the septum of which deviated to the left. Apart from the nervous system, no other abnormal physical signs were found.

Neurological Examination.—Mentally the patient was alert and cooperative. There was a slow hippus of both pupils, and a haziness in the outline of the temporal borders of both optic discs, accompanied by fulness of the veins, was noted. Otherwise the cranial nerves were normal. There was definite slight nuchal rigidity, but the presence of a Kernig's sign was questionable. Motor power and sense of position were normal, but a careful sensory examination revealed an area in the right lumbar region, supplied by the posterior branches of the 7th to 10th dorsal nerves, in which there was some diminution of sensation to pin prick. Definite tenderness to pressure over the erector spinae muscles was noted, and at times a mass contraction of these groups of muscles could be elicited by stroking the skin of the back. There was also tenderness along the costal margin and in the abdominal muscles on the right side. The deep reflexes throughout the body were moderately hyperactive, but could not be considered pathological. Babinski's sign was not obtained. The abdominal reflexes were at times readily elicited, but at other times were unobtainable in certain segments. Cremasteric reflexes were normal.

Course.—The spinal fluid, obtained on admission, was under normal pressure, was opalescent, and contained 720 mononuclear cells per c.mm. and an increased amount of globulin. Following lumbar puncture and the intravenous injection on two occasions of 50.0 cc. of a 50 per cent solution of glucose, the patient's headache diminished in severity and the nausea disappeared entirely. He was then able to enjoy and retain a normal diet, and his appetite rapidly returned to its usual excellence. The muscular tenderness continued for about 6 days gradually getting less, while the hypoesthesia over the back lasted for 3 weeks. During this time the patient occasionally complained of pain in a small area in each axilla, but these subjective phenomena were unassociated with abnormal physical findings. The temperature fell to normal within 48 hours after admission to the hospital; the pulse rate remained relatively slow, averaging between 60 and 70 per minute for the first 14 days, his normal rate being between 70 and 80. Some stiffness of the neck remained for 16 days. During the first 15 days in hospital, he experienced a good deal of abdominal distention, and his bowels did not move except with the aid of enemata, but after this time they moved spontaneously. Lumbar punctures were performed 4 times during the course of the illness, and on each occasion the amount of pleocytosis in the spinal fluid was less than that revealed by the preceding puncture. The patient was allowed to sit up and be out of bed 29 and 43 days, respectively, after the onset of illness. On Jan. 25, 2 days before he got up, the spinal fluid still contained 17 mononuclear cells per c.mm. and an increased amount of protein. On Feb. 3, 1935, 49 days after the onset of the illness, he was discharged at which time his physical examination was entirely negative except for some weakness induced by the long stay in bed. Following return to work he noticed some intention tremor of the right hand which lasted 3 months. On Sep. 20, 1935, the patient was free from symptoms and a neurological examination was negative.

liquefied clot were subsequently tested with potent virus, they were found to be susceptible.

A second specimen of blood was collected from the patient on admission to the Rockefeller Hospital. Clotting was prevented by a suitable amount of a sterile 0.1 per cent solution of heparin, and then the blood was used, within 2 hours of withdrawal, for the inoculation of 6 mice and a rabbit.

Mice.—Each of 6 mice received 0.03 cc. intracerebrally and 1.0 cc. intraperitoneally, respectively, of the heparinized blood. 2 mice died as a result of the inoculation and were discarded; 1 was found dead 3 days after inoculation, and its brain was removed for passage. After the brain was found to be free from ordinary bacteria, a 15 per cent emulsion was prepared and injected intracerebrally (0.03 cc.), intraperitoneally (0.12 cc.), and intranasally (0.02 cc.) into each of 5 mice. None of the animals became sick while under observation for 3 weeks and all of them were later shown to be susceptible to potent virus. Of the 6 original mice that were inoculated with blood, the 3 survivors never showed any evidences of illness while under observation for 4 weeks and later were found to be susceptible to potent virus.

Rabbits.—A rabbit received 0.25 cc. of the heparinized blood intracutaneously and remained well throughout the period of observation.

Evidence That the Active Agents Were Obtained from the Patients' Spinal Fluids

From the experiments described above we were led to believe that an active agent transmissible in mice had been obtained from the spinal fluid of W.E. and of R.E.S., and that the blood streams of the patients were free from demonstrable amounts of such agents at the time tests were made. Materials containing the active agents were free from ordinary bacteria, and they produced lesions in animals not unlike those caused by known viruses. In the paper that immediately follows (2) adequate evidence of the viral nature of the agents will be presented. Therefore, in the meantime, they will be spoken of as viruses for convenience. Furthermore, inasmuch as the active agents obtained from the spinal fluids of the two patients acted in a similar manner, we immediately suspected that they represented strains of the same virus or closely related viruses. This proved to be the case as will be shown by the evidence presented later in the paper.

In spite of our belief that the viruses were obtained from the spinal fluids of the patients, evidence had to be brought not only that such was the case but that, even though the viruses were in the spinal fluids, they were etiologically related to the disease from which the patients

tively, were injected intracerebrally and intraperitoneally into each of 5 normal mice. 4 of the animals became sick on the 7th day after incoulation. 3 of the sick animals were sacrificed and their brains, removed aseptically and found to be free from ordinary bacteria by means of cultures, were ground into a 10 per cent emulsion which was used for the inoculation of another 6 mice. At this time only intracerebral inoculations were performed, 0.03 cc. of the emulsion being used for each animal. All 6 mice were sick on the 7th day, and a bacteria-free emulsion of their brains caused sickness in still another group of normal mice. By means of intracerebral inoculation of, as a rule, a 10 per cent emulsion of sterile mouse brain, the active agent has been transmitted serially through 23 lots of mice.

A second specimen of spinal fluid, taken on Jan. 14, 1935, 1 month after onset, and containing 55 cells per c.mm., was inoculated intracerebrally (0.03 cc.) into 6 mice. None of the animals showed any symptoms during 17 days of observation. They were used in reinoculation experiments described later in the paper and were found to be susceptible to the active agent under investigation.

Rabbits.—A rabbit was inoculated intracutaneously with the spinal fluid collected from the patient on admission, but showed no symptoms during a period of observation lasting 3 weeks.

Blood.—The first sample of blood that was investigated was collected by Dr. TenBroeck at the onset of the patient's illness, 12 days before admission to the Rockefeller Hospital, and was stored in an ice box. After being on ice for 12 days some of the whole clotted blood was used for the inoculation of animals. Then the remaining material was separated into clot and serum, and the 2 portions were kept under vaseline seals in an ice box for 3 weeks at which time they were used for injections into animals.

Guinea Pigs.—Each of 2 guinea pigs received intracerebrally 0.1 cc. of an emulsion of the clotted blood that had been stored 12 days.[5] One of the animals showed no evidences of illness and was later found to be susceptible to potent virus. The other one had a slight amount of fever on the 4th and 5th days after inoculation and was sacrificed for passage. Of a sterile emulsion of this pig's brain, 0.1 cc. were given intracerebrally to one pig, and 1.0 cc. were injected into the subcutaneous tissues of the pads of the feet of another pig. Neither animal became sick.

Rabbits.—One rabbit received intracerebrally 0.1 cc. of an emulsion of the clotted blood stored for 12 days, while another animal was given 8.0 cc. subcutaneously. Neither rabbit became ill.

Mice.—After the clot, separated from the serum, had been stored for 3 weeks, it was found to have undergone liquefaction. Each of 12 mice received 0.03 cc. and 0.5 cc. intracerebrally and intraperitoneally, respectively, of the liquefied material. At the same time, 6 other mice received in a similar manner injections of the serum. None of the mice became sick, and, when the 12 that received the

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[5] Dr. Carl TenBroeck performed these inoculations.

liquefied clot were subsequently tested with potent virus, they were found to be susceptible.

A second specimen of blood was collected from the patient on admission to the Rockefeller Hospital. Clotting was prevented by a suitable amount of a sterile 0.1 per cent solution of heparin, and then the blood was used, within 2 hours of withdrawal, for the inoculation of 6 mice and a rabbit.

Mice.—Each of 6 mice received 0.03 cc. intracerebrally and 1.0 cc. intraperitoneally, respectively, of the heparinized blood. 2 mice died as a result of the inoculation and were discarded; 1 was found dead 3 days after inoculation, and its brain was removed for passage. After the brain was found to be free from ordinary bacteria, a 15 per cent emulsion was prepared and injected intracerebrally (0.03 cc.), intraperitoneally (0.12 cc.), and intranasally (0.02 cc.) into each of 5 mice. None of the animals became sick while under observation for 3 weeks and all of them were later shown to be susceptible to potent virus. Of the 6 original mice that were inoculated with blood, the 3 survivors never showed any evidences of illness while under observation for 4 weeks and later were found to be susceptible to potent virus.

Rabbits.—A rabbit received 0.25 cc. of the heparinized blood intracutaneously and remained well throughout the period of observation.

Evidence That the Active Agents Were Obtained from the Patients' Spinal Fluids

From the experiments described above we were led to believe that an active agent transmissible in mice had been obtained from the spinal fluid of W.E. and of R.E.S., and that the blood streams of the patients were free from demonstrable amounts of such agents at the time tests were made. Materials containing the active agents were free from ordinary bacteria, and they produced lesions in animals not unlike those caused by known viruses. In the paper that immediately follows (2) adequate evidence of the viral nature of the agents will be presented. Therefore, in the meantime, they will be spoken of as viruses for convenience. Furthermore, inasmuch as the active agents obtained from the spinal fluids of the two patients acted in a similar manner, we immediately suspected that they represented strains of the same virus or closely related viruses. This proved to be the case as will be shown by the evidence presented later in the paper.

In spite of our belief that the viruses were obtained from the spinal fluids of the patients, evidence had to be brought not only that such was the case but that, even though the viruses were in the spinal fluids, they were etiologically related to the disease from which the patients

were suffering. The necessity that such evidence must be sought was emphasized by the facts (*a*) that Theiler (3) in 1934 described a spontaneous virus disease in mice characterized by paralysis of the extremities, (*b*) that Traub (4) in 1935 reported that he was able by the injections of inert materials into the brains of normal looking stock mice to demonstrate the presence of a latent virus capable of causing neurological symptoms similar to those shown by our sick mice, and (*c*) that Flexner (5) obtained the virus of herpes simplex from the spinal fluid of a patient being treated for meningo-vascular syphilis, but showing no evidence of acute involvement of the central nervous system.

A spontaneous virus disease lying dormant in a colony of mice may manifest itself by the occasional presence of a sick animal or by the presence of a certain number of immune individuals. With regard to the first contingency, if the virus is inactive in the nervous system until activated by some insult to the brain, then symptoms should be induced by the intracerebral injections of a variety of materials. With regard to the presence of immune mice in the colony, there are two possibilities. In the first place, a virus may not be entirely inactive and may cause a very mild disease from which recovery resulting in immunity takes place. Secondly, it is conceivable that inoculations of inert or noninfectious materials may activate a latent virus to such an extent that it will produce a subclinical infection sufficient to render an animal resistant to potent virus administered subsequently. In either case immune animals should be encountered in a fortuitous manner among the mice used either for primary inoculations or for reinoculations. The results of many experiments, briefly presented below, clearly show that neither illness caused by a virus similar to ours nor immunity to it was fortuitously encountered in the stock mice during the period, 9 months, of investigation.

During this period we have had the opportunity of examining materials from 23 patients by means of mouse inoculations. The patients presented a variety of clinical pictures; 7 of them fell under the clinical diagnosis of "acute aseptic meningitis," 6 belonged to the group of encephalitides that follow infectious diseases or vaccination (measles 2, German measles 2, mumps 1, antirabic vaccination 1), 2 had encephalitis (1 Economo's and 1 unclassified), 2 had an unclassified lymphocytic meningitis, 2 had multiple sclerosis, 1 had herpes zoster, 1 had acute poliomyelitis, 1 had an ascending paralysis of the Landry type, and 1 had no recognizable disease of the nervous system.

In the course of this study we have inoculated 221 mice intracerebrally with materials, *e.g.*, spinal fluid, blood, brain, derived directly from the patients. Into 111 more mice we have injected intracerebrally materials, *e.g.*, brain emulsions, blood, spinal fluid, derived from the animals originally inoculated from the patients. In none of the 332 mice so inoculated were any symptoms of disease produced. After an interval of 3 or more weeks, 237 of them were reinoculated intracerebrally with potent virus and without exception were found to be susceptible.

The failure of the mice to become sick after the inoculation of materials from such a variety of clinical conditions indicates that the mice that became ill following intracerebral inoculations of spinal fluid from W.E. and R.E.S. did so because of the presence of a virus in the fluids.

Further evidence that we obtained the virus from the spinal fluid of W.E. and R.E.S., instead of picking it up from the mice used in the work, is afforded by the solid immunity exhibited by the mice that received injections of their spinal fluid, became sick, and recovered, or developed no definite signs of illness. In the reinoculation test for immunity, described below, in the mice that had received W.E.'s spinal fluid and had recovered after an illness, we included several other groups of mice, some of which had been given, several weeks previously, materials known to contain virus and others, such as normal stock mice, that had never been inoculated. A similar procedure was followed with R.E.S.'s mice and virus.

The following groups of mice were injected intracerebrally (0.03 cc.) with potent W.E. virus contained in an emulsion prepared from an infected mouse brain: (*a*) 2 mice that had recovered from an illness produced by intracerebral injections of spinal fluid removed from W.E. on admission to the hospital; (*b*) 6 mice that had received intracerebral injections of blood taken from the patient on admission and had exhibited no signs of illness; (*c*) 5 mice that had been inoculated intranasally with an emulsion of a virus-infected mouse brain, and had shown no evidences of sickness; (*d*) 4 mice that had been injected intraperitoneally with an emulsion of liver taken from an infected mouse and had remained without signs of illness while under observation; (*e*) 5 normal stock mice. All animals that had received W.E.'s spinal fluid or tissues known to contain active virus were found upon reinoculation with potent virus to be immune, while none of the normal mice or mice that had received W.E.'s blood was resistant (Table I).

The following groups of mice were injected intracerebrally (0.03 cc.) with potent R.E.S. virus contained in an emulsion prepared from an infected mouse brain: (*a*) 5 mice that had been inoculated intracerebrally with spinal fluid obtained from

R.E.S. on admission to the hospital 12 days after onset of disease, and had shown no signs of illness: (*b*) 6 mice that remained well after receiving intracerebral injections of R.E.S.'s spinal fluid collected 18 days after admission to the hospital 30 days after onset of illness; (*c*) 11 mice that had been injected with blood taken from R.E.S. at the onset of illness, 12 days before admission, and had remained

TABLE I

Results of Reinoculation Experiment Supporting Other Evidence That the Viruses Were Obtained from W. E. and R. E. S.'s Spinal Fluid

Work with W. E. virus			Work with R. E. S. virus		
Original inocula	Reinoculated, intracerebrally, with W. E. virus		Original inocula	Reinoculated, intracerebrally, with R. E. S. virus	
	Sick	Dead		Sick	Dead
Admission spinal fluid, W. E.	0/2	0/2	Admission spinal fluid, R. E. S.	0/5	0/5
Admission blood, W. E.	6/6	5/6	Spinal fluid taken 4 wks. after onset, R. E. S.	6/6	5/6
Infected mouse brain, intranasally	0/5	0/5	Blood taken at onset, R. E. S.	11/11	9/11
Infected mouse liver, intraperitoneally	0/4	0/4	Admission blood, R. E. S.	3/3	0/3
None, control	5/5	4/5	Infected mouse liver, intraperitoneally	0/5	0/5
			Infected mouse liver, subcutaneously	0/5	0/5
			None, control	6/6	2/6

The results of the experiment are expressed as fractions, the denominators representing the number of mice inoculated, the numerators the number of mice either sick or dead.

well; (*d*) 6 mice that had received blood taken from R.E.S. on admission and had remained well; (*e*) 5 mice that had been inoculated intraperitoneally with virus-infected mouse liver and had shown no signs of illness; (*f*) 5 mice that had received subcutaneous injections of virus-infected mouse liver and had remained well; (*g*) 6 normal mice. All of the mice that had received R.E.S.'s original spinal fluid

or tissues known to contain virus were found upon reinoculation with potent virus to be immune, while none of the normal mice or mice that had been injected with R.E.S.'s blood or second spinal fluid was resistant (Table I).

The results of the reinoculation experiments just described, and summarized in Table I, clearly show that immune mice were not encountered in a fortuitous manner; all of the mice in each group were either resistant or susceptible. The results of many other reinoculation experiments and investigations in which numerous primary injections have been made, attest the fact that our stock mice are free from the virus under investigation. Consequently, one can look upon the results of the experiments shown in Table I as additional evidence that the virus with which we are now working was present in the spinal fluid of W.E. and R.E.S. on admission to the hospital, that it was not present in demonstrable amounts in R.E.S.'s spinal fluid 18 days after admission, 30 days after the onset of illness, and that it was not demonstrable in the blood of either patient upon admission.

Evidence That the W.E. and R.E.S. Viruses Are Identical

Even from the brief descriptions that have been given of the action of the viruses obtained from W.E. and R.E.S., it might be suspected that they are identical or closely related. Furthermore, the clinical pictures presented by the patients were sufficiently similar to make it inevitable that we should recognize the possibility of a close relation between the causal agents and immediately attempt to determine whether such was the case.

5 mice, that had been inoculated with W.E.'s virus and had recovered, were then reinoculated with the same strain of virus and found to be immune. Following this, the animals were again reinoculated. Upon this occasion, R.E.S. virus was administered intracerebrally, and 5 normal stock mice were also inoculated in a similar manner as controls. 5 mice that had been shown by means of reinoculations to be immune to R.E.S.'s virus and 6 normal stock mice (controls) received intracerebrally injections of a 10 per cent emulsion prepared from a mouse brain known to contain potent W.E. virus.

The results of the above experiments, summarized in Table II, show that all of the 11 control mice became sick in a characteristic manner, and 4 of them died, while none of the mice immune to the homologous strain of virus became ill as a result of injections of the

heterologous strain of active agent. This type of experiment has been repeated on several occasions with similar results and shows clearly that the W.E. virus is identical or closely related to the R.E.S. strain. Further immunological evidence that the 2 strains are of an identical nature can be found in the results of the experiments on cross-neutralization presented later in this paper (Table III) and in the communication that immediately follows (2).

TABLE II

Results of Experiment to Test by Reinoculation the Immunological Relation of the W. E. to the R. E. S. Virus

Condition of mice	Reinoculated with R. E. S. virus		Condition of mice	Reinoculated with W. E. virus	
	Sick	Dead		Sick	Dead
Immune to W. E. virus	0/5	0/5	Immune to R. E. S. virus	0/5	0/5
Normal, control	5/5	2/5*	Normal, control	6/6	2/6*

The results of the experiment are expressed as fractions, the denominators representing the number of mice inoculated, the numerators the number of mice either sick or dead.

* Remainder sacrificed for passage virus.

Evidence That the Virus Found in the Spinal Fluids Was Etiologically Related to the Disease of the Patients

It is conceivable that the virus in the spinal fluids was not the cause of the disease of the patients but appeared there accidentally. If that were true, at least two possibilities regarding the presence in the patients of humoral neutralizing antibodies against the virus would be obvious. In the first place, if the virus did not multiply sufficiently in the body of the hosts, or if it caused no reaction, then one might expect to find no humoral antibodies in our patients either during the illness or in convalescence. If, on the other hand, the virus, having caused an infection in the hosts at some previous time, had persisted in them to appear accidentally in the spinal fluids, one would expect to find humoral antibodies against it at the very beginning of the illness as well as during convalescence. In view of the

fact that we know of no phenomenon similar to the Weil-Felix reaction in connection with virus diseases, we assume that the absence of antiviral antibodies at the onset of an illness and their appearance during convalescence are of some significance as regards the etiology of a disease under investigation. With these ideas in mind we looked for the presence of neutralizing antibodies in samples of blood collected from the patients early in the course of their illness and during convalescence.

Before describing the neutralization experiments, it is necessary to state that guinea pigs are highly susceptible to the virus administered either intracerebrally or subcutaneously, almost all of the pigs dying upon injection of the active agent by either route. The details of this work appear in the paper that follows immediately (2). Inasmuch as it was easier to interpret the results of neutralization tests when the mixtures of virus and serum were injected into young guinea pigs instead of mice, the former animals were used in much of this kind of work.

A 10 per cent emulsion of a virus-infected guinea pig brain free from ordinary bacteria was prepared with a diluent consisting of 10 per cent human ascitic fluid in Locke's solution, and then centrifuged at full speed for 15–20 minutes. Serial 10-fold dilutions of the supernatant fluid were made with the diluent described above, the highest dilution being 10^{-3}. 0.25 cc. of each dilution of virus, ranging from 10^{-1} to 10^{-3}, were mixed with an equal amount of each sample of serum under investigation and allowed to stand at room temperature for 5 or 6 hours. Then each mixture, 0.5 cc., was injected subcutaneously into a separate guinea pig. Daily observations of the guinea pigs including the recording of temperatures were made for 21 days or until the animals died.

The results of the experiment summarized in Table III show that the serum from neither patient possessed neutralizing antibodies at the onset or early in the course of the disease, while sera from both patients neutralized at least 100 lethal doses of the virus 9 to 11 weeks later. The experiment was repeated with the same samples of serum as well as with specimens collected from the patients at different times. The fact that antibodies against the virus were not present early in the course of the disease but were found during convalescence has been interpreted by us as indicating that the virus was etiologi-

cally related to disease of the patients from whose spinal fluids it was obtained.

It is important to note that the antibodies were demonstrated in samples of blood taken well along in convalescence, 9th and 11th weeks, respectively. An attempt was made to demonstrate antibodies in R.E.S.'s serum 6 weeks after the onset without much success. It appears, therefore, that antibodies against the virus may develop slowly and that premature tests may fail to reveal their presence which would be demonstrable at a later date.

TABLE III

Results of Neutralization Tests Conducted with Samples of Serum Taken from W. E. and R. E. S. Early in the Course of Illness and During Convalescence

Dilution of virus	Tests with W. E. serum		Tests with R. E. S. serum	
	Serum taken Dec. 23, 1934, 9 days after onset	Serum taken Mar. 7, 1935, 11 weeks after onset	Serum taken Dec. 15, 1934, at onset	Serum taken Feb. 19, 1935, 9 weeks after onset
10^{-1}	Died	No fever, survived	Died	Typical illness, died
10^{-2}	"	Transient fever, survived	"	No fever, survived
10^{-3}	Not done	No fever, survived	"	" " "

Tests were conducted in the manner described in the text. An emulsion of guinea pig brain infected with W. E. virus was used for the tests with the samples of serum from both patients, W. E. and R. E. S.

DISCUSSION

Inasmuch as the results of experimental studies on the character of our active agent and a comparison of its characteristics with those of other known viruses appear in the paper that immediately follows (2), and since the question of the distribution of the virus and its relation to disease in man and lower animals is also considered in the same paper, a general discussion has been reserved for that communication. At this time, however, it seems advisable to call attention to several interesting facts. Most of the spontaneous virus diseases of the human central nervous system that have been described in the past seem primarily to attack the substance of the brain and cord and to involve the meninges, if at all, in a secondary manner. In the cases that we have described, the history and clinical findings

point to the fact that the main part, if not all, of the insult to the central nervous system was borne by the meninges.

In investigations of neurological maladies, it is natural that the spinal fluid should come in for its share of interest and that many attempts should have been made to discover viruses in it responsible for the particular diseases being studied. Only upon rare occasions has a virus been isolated from the spinal fluid. In certain instances, the evidence of the viral nature of the agent obtained has not been adequate; in others, definite viruses have been isolated, but no conclusive evidence has been presented that they were present in the spinal fluid instead of contaminating blood, or that their presence was of etiological significance instead of an incidental finding. In the first group can be placed Kobayashi's work (6) on the Japanese type B encephalitis and Eckstein's (7) investigations on serous meningitis. In the second group belong the experiments of Gildemeister (8) and Eckstein, Herzberg-Kremmer, and Herzberg (9) on encephalitis following vaccination against smallpox, and, as pointed out by Flexner (5), all the work on epidemic or lethargic encephalitis in which herpes simplex virus was isolated. It is possible that Breinl (10) obtained a virus of etiological significance from the spinal fluid of a patient with Australian X disease, but he did not carry it further than the first generation in experimental animals (monkeys). Reports in the literature fail to provide definite evidence that a virus can exert most, if not all, of its harmful action against the meninges of the human brain and cord and be found in the spinal fluid of an infected individual, but we believe that sufficient evidence has been presented in the present paper to convince one that such a virus disease of man exists.

SUMMARY AND CONCLUSIONS

The clinical pictures presented by two men suffering from an acute bacteria-free lymphocytic meningitis, and the method of obtaining a virus from their spinal fluids were described. Evidence was then adduced to show that the virus was really in the spinal fluids, that the strains of virus obtained from the two patients were identical or closely related, and that the active agent in the spinal fluids was etiologically significant.

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MENINGITIS IN MAN CAUSED BY A FILTERABLE VIRUS

II. Identification of the Etiological Agent

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Plates 30 and 31

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In the preceding paper (1) we described the clinical picture presented by two patients who were suffering from a nonbacterial lymphocytic meningitis, and the method by which a virus-like agent was isolated from each patient's spinal fluid. Then we showed that the two agents were immunologically identical and that they were etiologically related to the disease process in the individuals from whom they were obtained. In the present communication we shall describe experiments in support of the viral nature of the agent, state details concerning the range of susceptible hosts and the clinical and pathological picture developed in each, compare our active agent with known viruses that spontaneously affect the central nervous system of man or lower animals or that might have contaminated our materials because of their proximity in the laboratory, and, finally, discuss the relative importance of our agent as a cause of disease in human beings.

Viral Nature of the Transmissible Agent

Invisibility.—Numerous sections of organs from animals dead of infection with the active agent have been stained according to Giemsa's method and studied by means of the microscope. In no instance has it been possible to demonstrate the presence of ordinary bacteria, protozoa, or fungi.

Failure of Cultivation.—Infectious material was seeded in meat infusion broth and on blood agar. The cultures were then incubated either aerobically or anaerobically at 37°C. for 2 weeks. With the exception of a few obvious contaminants no organism of etiological significance was encountered.

Filterability.—Both strains of the active agent were tested by means of Seitz filters and graded collodion membranes. Inasmuch as the findings with the 2 strains were the same and since only the W. E. strain was tested with Berkefeld

candles, the results of experiments on the filterability of this strain will be presented in full.

Filtration through Berkefeld Candles.—3 Berkefeld candles, V, N, and W, shown by air pressure to be free from leaks, were satisfied by the passage of 40 cc. of sterile broth through each of them.

A 2 per cent emulsion of mouse brains infected with the W.E. strain was prepared in a diluent of 10 per cent human ascitic fluid in equal parts of nutrient broth and Locke's solution. After thorough centrifugation at 2000 R.P.M., the supernatant fluid was removed and divided into 4 equal parts of about 15 cc. each.

1 portion of the supernatant fluid was passed through the V candle, and 0.03 cc. of the filtrate were then inoculated intracerebrally into each of 6 mice. 50 per cent of the mice died on the 7th day after inoculation and the remainder were sacrificed for passage. When the brains of the animals were found to be free from ordinary bacteria, a 10 per cent emulsion in Locke's solution was prepared from which decimal dilutions were made. Intracerebral inoculations into mice of dilutions up to 10^{-2} caused death in 100 per cent of animals after the usual incubation period.

The 3 remaining portions of the supernatant fluid, purposely contaminated with *B. prodigiosus*, were passed through the candles, 1 through the V used above, 1 through the N, and 1 through the W. Portions from each filtrate were separately cultured aerobically on blood agar and aerobically and anerobically in meat infusion broth. Portions from each filtrate were also inoculated intracerebrally into mice. The cultures of the filtrates showed no evidence of bacterial growth after incubation at 37°C. for 7 days. However, all the mice died within 8 to 12 days after inoculation.

From the results of the experiment just described it is obvious that the active agent passes Berkefeld candles V, N, and W that hold back ordinary bacteria.

Filtration through Seitz Filters.—A 2 per cent emulsion of mouse brains infected with the W.E. strain was prepared in a diluent consisting of equal parts of human ascitic fluid, nutrient broth, and sterile distilled water. The emulsion was centrifuged at 1500 R.P.M. for 30 minutes and the supernatant fluid was removed, a portion of which was reserved for titration of its potency. 70 cc. of the remaining supernatant fluid was forced, under 8 pounds of pressure, through a 60 mm. Seitz pad which had been satisfied by the passage through it of 100 cc. of sterile broth. A portion of the filtrate was reserved for titration of its potency, while the remainder was used for filtration through graded collodion membranes to be described later.

The ability of the active agent to pass through Seitz pads was estimated by a comparison of the results of the intracerebral titrations

of the unfiltered and filtered portions of the supernatant fluid. In the titrations, not only were the morbidity and mortality rates considered, but the end-points were more precisely determined by tests for the presence of immunity in the mice surviving the original inoculations. As is evident from the results shown in Table I, the active agent was present in dilutions of the unfiltered material up to 10^{-5} because 3 of 5 mice receiving inoculations of this dilution were immune, while demonstrable amounts of the agent were not present in dilution of the filtrate higher than 10^{-2}. Such results clearly indi-

TABLE I

Results of Filtration of W. E. Strain of Virus through a Seitz Filter

Titration of virus before filtration					Titration of virus after filtration				
Dilution of virus	Results of initial inoculation		Results of reinoculation with potent virus		Dilution of virus	Results of initial inoculation		Results of reinoculation with potent virus	
	Sick	Dead	Sick	Dead		Sick	Dead	Sick	Dead
Undiluted	6/6	3/6	Not done		Undiluted	6/6	1/6	1/6	0/6
10^{-1}	6/6	5/6	"	"	10^{-1}	6/6	2/6		
10^{-2}	6/6	5/6	"	"	10^{-2}	6/6	1/6	0/5	0/5
10^{-3}	6/6	4/6	"	"	10^{-3}	4/6	0/6	3/3	2/3
10^{-4}	1/6	1/6	1/4	1/4	10^{-4}	0/6	0/6	6/6	6/6
10^{-5}	0/6	0/6	2/5	2/5	10^{-5}	0/6	0/6	6/6	6/6
Uninoculated control mice			6/6	6/6	Uninoculated control mice			6/6	6/6

The results of the experiment are expressed as fractions, the denominators representing the number of mice inoculated, the numerators the number of mice either sick or dead.

cate that the agent was capable of passing a Seitz pad in spite of the fact that 99.9 per cent of it was held back under the conditions of the experiment.

Filtration through Graded Collodion Membranes.—As more accurate information regarding the approximate size of particles can be obtained by filtration through collodion membranes of graded average pore diameter than through Berkefeld candles or Seitz pads, such membranes were employed in the investigation of the approximate size of our active agent.

For filtration through membranes[1] of average pore size ranging from 850 mμ

[1] Experiments on filtration through graded collodion membranes were performed with the cooperation of Dr. J. H. Bauer of the International Health Division of the Rockefeller Foundation.

to 250 mμ, the supernatant fluid (with the active agent) described for the experiment with Seitz filters was employed. For membranes of average pore diameter ranging from 250 mμ to 50 mμ, the Seitz filtrate of that supernatant fluid was used. Before filtration each membrane was satisfied by the passage of 6 cc. of broth through it. The material was forced through the 850 mμ membrane under a pressure of 10 pounds, while a pressure of 30 pounds was used for the other membranes. The filtrate from each membrane was tested for the presence of the infectious agent by means of intracerebral inoculations into white mice, both the results of primary inoculations and those of reinoculations for tests of immunity in the survivors being taken into consideration.

TABLE II

Results of Filtration of W. E. Strain of Virus through Collodion Membranes

Average pore size of membrane		Results of inoculation with membrane filtrates		Results of reinoculation with potent unfiltered virus	
		Sick	Dead	Sick	Dead
	mμ				
Unfiltered supernatant used for filtration	850	6/6	3/6	Not done	
	650	6/6	4/6	" "	
	450	6/6	5/6	" "	
	250	6/6	3/6	" "	
Seitz filtrate used for filtration	250	4/7	1/7	4/6	4/6
	210	6/6	1/6	4/5	3/5
	150	0/6	0/6	6/6	6/6
	110	0/6	0/6	6/6	6/6
	85	0/6	0/6	6/6	6/6
	50	0/6	0/6	6/6	6/6
Uninoculated control mice				6/6	6/6

The results of the experiment are expressed as fractions, the denominators representing the number of mice inoculated, the numerators the number of mice either sick or dead.

The results of the above experiment, summarized in Table II, indicate that all or some of the mice receiving filtrates from membranes with an average pore diameter of 210 mμ or greater became sick or died. When the survivors were reinoculated it was found (Table II) that immunity was present in some of the animals that had received filtrates from the membranes with average pore diameters of 250 mμ and 210 mμ but not in those inoculated with filtrates

from membranes of 150 mμ or smaller average pore diameters. According to Elford (2), particles, in order to pass through a membrane with pores having an average diameter of 210 mμ, cannot possess a diameter greater than one-half or three-quarters of that of the pores. Consequently, the particles of our active agent do not possess diameters greater than 100–150 mμ.

Results of experiments being conducted at the present time in which Berkefeld V filtrates, instead of Seitz filtrates, containing the active agent are passed through graded collodion membranes indicate that our virus is capable of penetrating membranes with average pore diameters of 150 mμ. V candles allow the passage of more virus than do Seitz pads, and, inasmuch as the estimation of the size of a virus is influenced by its concentration in the material filtered, the difference in the results of the experiments cited is adequately accounted for. Our active agent is obviously smaller than any known bacterium, and results of experiments in which attempts have been made to estimate its actual size will be given in a later paper.

Preservation of the Active Agent in Glycerol.—In infected mouse brains stored in 50 per cent glycerol in Locke's solution at 0°C., sufficient virus remained active for 217 days to kill 3 of 5 mice inoculated intracerebrally with a 10 per cent emulsion of them. Under similar conditions the virus in the brains of guinea pigs retained its activity for 235 days.

Preservation of the Active Agent in the Absence of Glycerol.—Virus was easily demonstrated in a 10 per cent emulsion made from infected mouse brains and kept under a vaseline seal at +5°C. for 56 days. Virus in a guinea pig brain stored in a Petri dish at +5°C. for 32 days was still active.

Preservation of the Active Agent by Means of Desiccation.—To a 10 per cent emulsion of infected mouse brains in Locke's solution sufficient sterile acacia was added to make a 2.5 per cent solution. This mixture was dried in the frozen state, sealed *in vacuo* (3), and stored at +4°C. for 49 days. Sufficient active virus was still present at the end of this time to kill 1 of 4 mice and immunize the other 3 when small amounts of the dried material resuspended in Locke's solution were inoculated intracerebrally.

The facts presented above regarding the invisibility, failure of cultivation, filterability, size, and preservation of our active agent clearly indicate that it should be classed with the viruses. Now we shall pass to a consideration of the clinical and pathological pictures produced by the virus in susceptible laboratory animals.

Response of Laboratory Animals to Inoculations of the Virus

Mice.—The following events take place in mice as a result of intracerebral inoculations of the virus.

Clinical Picture.—During the first 5 days after inoculation the mice appear well. Occasionally on the 5th, but more commonly on the 6th day, symptoms appear, at which time some of the mice may be found dead although none of them were obviously sick on the preceding day, while others with dirty, ruffled fur, half-closed eyes, and hunched backs remain motionless. When disturbed they occasionally leap up and down in the jar and fall over backwards; but the characteristic reaction, especially when the animals are susupended by the tail, is for them to exhibit coarse tremors of the head and extremities frequently going on to a series of clonic convulsions terminating in a tonic extension of the hind legs. In male mice an erection sometimes occurs during the convulsions. The convulsions, often the cause of death, may also occur spontaneously either in sick mice or even in those that appear to be normal. As a rule, the animals either die within 1 to 3 days after the onset of symptoms or quickly recover in 5 or 6 days. Paralyses have never been observed.

Pathological Picture.—Brain and Cord. In this strain of mice, only a slight congestion of the brain is observed in the gross. Stained sections reveal an engorgement of the surface vessels and an occasional hemorrhage in the meninges. The meninges of the brain, and to a less extent those of the cord, are infiltrated with mononuclear cells resembling lymphocytes (Figs. 1 and 2). At times a slight infiltration of mononuclear cells into the subependymal tissues and the choroid plexus is noted. Necrosis of nerve cells and perivascular cuffing are rarely if ever found in early lesions. If the animals are chronically ill or if they have been killed 2 to 3 weeks after recovery considerable perivascular cuffing may be present. *Liver and Spleen.* Sections of the liver reveal some engorgement of the capillaries, a definite increase in the number of Kupffer cells, and a few small areas of focal necrosis. Nothing particularly characteristic of the disease is seen in the spleen. *Lungs.* Regardless of the portal of entry of the virus, the lungs frequently show areas of discoloration and consolidation which are free from bacteria. Stained sections of these areas reveal an interstitial bronchopneumonia similar to that caused by a number of viruses. The small blood vessels are surrounded by numerous round cells. Involvement of the bronchioles, characterized by desquamation of the mucous membrane and cuffing with mononuclear elements, occurs only where the pathological changes are most marked. In definitely consolidated portions of the lungs, the alveolar walls are densely infiltrated with mononuclear cells; the alveoli may be collapsed or may contain an exudate consisting of fibrin and a few cells which are usually of the mononuclear group. In some sections lungs present nothing more than distended capillaries and a few alveoli filled with red blood cells. *Other Organs.* The remaining organs present no characteristic changes.

Distribution of the Virus in the Mouse.—After a number of experiments in which blood and emulsions of organs from infected mice were inoculated into normal mice it was obvious that the virus could be recovered from the blood and hence equally well from the brain, liver, spleen, or lungs.

Effect of Route of Inoculation upon the Course of the Disease.—The course of events varied with the route of inoculation of the virus. (*a*) When the virus was introduced intracerebrally, the typical illness described above resulted 5 to 7 days after inoculation. (*b*) When 0.5 cc. of a virus-containing emulsion were administered intraperitoneally, the mice showed indefinite symptoms of illness after an incubation period of 8 days, and only a few died. When the liver of one of the sick animals was removed and an emulsion of it was injected intraperitoneally into normal mice, the animals did not become sick, but were later found to resist infection by the virus administered intracerebrally. (*c*) Subcutaneous inoculations of the virus produced no obvious illness in mice but immunity to virus given intracerebrally. (*d*) Virus administered intranasally to etherized mice resulted in no illness but solid immunity. (*e*) Intravenous injections of the virus in the form of a 10 per cent suspension prepared from an infected brain produced no evidence of illness, but, when an emulsion of the brains of these inoculated mice, removed 12 days after the injections, was administered intracerebrally to normal mice, the typical picture of the disease developed and the virus could be passed from them to another group of normal mice.

Contagiousness of the Disease.—Traub's (4) report of the discovery of a virus, later shown to be closely related to if not identical with our active agent, that was widely disseminated in his stock mice and which presumably spread in some manner from mouse to mouse, induced us to ascertain whether either infection or immunity could be produced in normal mice by contact with a mouse infected with our virus, precautions being taken when the infected animal died to prevent the normal contacts infecting themselves intranasally by devouring the corpse. 2 experiments were performed in the following manner. A mouse inoculated intracerebrally 4 days previously with the R.E.S. strain of virus was placed in a jar with 5 normal mice 4 weeks of age. The inoculated animal became sick on the 1st day after being placed with the normal mice, the 5th day after inoculation. It lived 3 days longer and was found dead on the morning of the 4th day. The normal contacts were observed for 3½ weeks and no evidences of illness were noted. Then they were inoculated intracerebrally with potent virus and found to be fully susceptible. The second experiment was conducted with the W.E. strain of virus in a manner similar to that just described. No infection or immunity was produced by close contact of the normal mice with the infected animal. The results of the two experiments clearly indicate that our virus does not spread with ease from mouse to mouse when normal animals are not allowed to devour an infected corpse.

Inheritance of Resistance to the Virus.—On two occasions, 4 weeks old mice from infected or recovered mothers have been inoculated intracerebrally with potent virus. In each instance all the animals became sick on the 5th or 6th day after

inoculation and were dead by the 7th. Thus it appears that no immunity to the virus is to be found in mice born of infected or immune mothers.

Guinea Pigs.—Having presented in detail the reactions of mice to the virus, we shall now describe those of guinea pigs.

Susceptibility.—Guinea pigs inoculated intracerebrally (0.1 cc.) or subcutaneously (0.25 cc.) with emulsions of infected mouse brains become sick and the majority of them die. The virus can be propagated indefinitely in this host by means of brain to brain or by brain to skin to brain passages.

Clinical Picture.—The clinical picture produced in guinea pigs by intracerebral inoculations of the virus is essentially the same as that caused by subcutaneous injections, with the exception that the former method of administration leads to a more acute illness. After intracerebral inoculation, the temperature of the pigs usually becomes elevated within 24 to 48 hours, while after subcutaneous administration the rise is, as a rule, delayed 3 to 6 days. The temperature once elevated usually remains high, often reaching 106–107°F., until shortly before death when it may drop suddenly to a subnormal level. The average duration of illness following intracerebral and subcutaneous inoculations is 9 or 10 and 12 to 16 days, respectively. When infected, the pigs lose weight rapidly and may develop labored breathing and a mild diarrhea. Marked terminal salivation is not unusual. Definite neurological manifestations are not observed.

Pathological Picture.—A mild meningeal reaction characterized by infiltration of mononuclear cells and an interstitial bronchopneumonia (Figs. 3 and 4) are found in pigs intracerebrally inoculated with the virus. Little or no pathological change has been seen in the brain and cord. After subcutaneous inoculations, the picture is similar to that just described with the exception that there is a minimum involvement of the meninges.

Distribution of Virus in the Body.—The blood of guinea pigs inoculated intracerebrally is infectious for mice at least as early as the 4th day after inoculation and remains so until the death of the animal. The brains of pigs that die as the result of subcutaneous administration of the virus are infectious for mice. Other organs were not tested, but the fact that the active agent is in the blood indicated that emulsions of all organs would also contain it.

Monkeys.—Although monkeys are susceptible to the virus, we have not made extensive investigations of its effects in them. The animals become sick after intracerebral inoculations and the virus can be propagated by brain to brain passages.

Clinical Picture.—4 to 7 days after intracerebral inoculations of 1.0 cc. of a 10 per cent emulsion of infected mouse brains, the monkeys develop fever, the temperature being 104°F. or more, that lasts 1 to 3 days. When the fever disappears the animals may begin to look sick, lose their appetite, and become less active.

One monkey appeared to develop a hyperesthesia, inasmuch as it sat in the cage on the smallest possible area of body surface and refused to be touched by its mate. Recovery usually takes place and is complete within about 3 weeks. A cisternal puncture was performed on 1 monkey 9 days after inoculation, and the cerebrospinal fluid contained 1410 cells of which 97 per cent were mononuclear elements. Serum from recovered animals contains neutralizing antibodies.

Pathological Picture.—The only pathological changes of importance were found in the brain and cord. In the gross, the surface of these organs and the choroid plexus appear pinker than normal. Stained sections reveal a moderate amount of mononuclear infiltration in the meninges. There is also a marked involvement of the choroid plexus, a phenomenon either not observed or decidedly less definite in mice and guinea pigs. Between the walls of the blood vessels and the ependymal covering of the plexus occurs a dense infiltration of mononuclear cells (Figs. 5, 6, and 7). In places, the collection of cells causes sufficient tension to distort the cuboidal ependymal cells into long tenuous elements difficult to see (Fig. 7). There is a moderate amount of infiltration in the subependymal tissues and an occasional blood vessel is surrounded by a single layer of mononuclear elements. As yet, no lesions of importance have been found in the nervous system proper except in sections through or near the site of inoculation.

Distribution of Virus in the Body.—We have found that the brain, spinal fluid, and blood of a sick monkey contain the virus.

Rabbits.—Both strains of the virus were administered intracerebrally, dermally, and intradermally to several rabbits. No obvious illness was caused in the animals and the matter was not pursued.

From the data, it appears that mice, guinea pigs, and monkeys are susceptible to our virus and that it can be serially passed in them with ease, while rabbits show little or no susceptibility. The susceptible hosts display differences in their clinical symptoms but a similarity in the pathological pictures. The route of inoculation is sometimes of importance, because in the mouse clinical symptoms of the characteristic disease occur only after intracerebral administration of the virus, while in pigs both intracerebral and subcutaneous injections are equally efficacious. The most marked pathological changes are found in the meninges, choroid plexus, and the lungs. We have not as yet been able to demonstrate inclusion bodies in infected cells similar to those described by Traub (4).

Identification of the Virus

To identify our virus a comparison of its properties with those of a selected group of known viruses was made. The selection for com-

parison was conducted in the following manner. First, any virus that had been investigated in our laboratory or in other laboratories at the Institute in New York City or at Princeton was considered as a possible cause of the infection in the patients or a subsequent contaminant of our animal material. Secondly, any virus which, from its description, has characteristics closely resembling those of our active agent was critically examined in order to confirm or dispose of their identity.

Viruses Being Studied or Carried in Our Laboratory.—Virus III of rabbits, and the viruses of vaccinia, herpes simplex, infectious myxomatosis of rabbits, mumps, psittacosis, louping ill, and Rift Valley fever were for obvious reasons easily disposed of. Moreover, a known immune louping ill serum failed to neutralize our virus.

Viruses under Investigation in Other Laboratories of The Rockefeller Institute in New York City and Princeton.—B virus and the viruses of pseudorabies, equine encephalomyelitis, poliomyelitis, rabies, yellow fever, vesicular stomatitis, human influenza, swine influenza, lymphogranuloma inguinale, St. Louis type of encephalitis, Theiler's disease of mice, and rabbit pox were obviously not closely related to our active agent. Furthermore, convalescent serum from the two patients did not neutralize the virus of the St. Louis type of encephalitis.

Traub (4) recently described a virus indigenous to the stock mice in The Rockefeller Institute at Princeton which causes a malady indistinguishable from that induced by our active agent and produces pathological changes similar to those seen in our animals, with the exception that a slight amount of necrosis of nerve cells and a few intranuclear inclusion bodies were reported to occur in his infected animals.

Reports in the Literature of Viruses Similar to Ours.—As far as could be ascertained from the literature only one other virus, in addition to that described by Traub and noted above, has characteristics closely resembling those of our active agent. This virus was discovered by Armstrong and Lillie (5) and designated by them as the virus of lymphocytic choriomeningitis. Its properties, host range, and activities appear to be identical with those of our virus.

It is a well recognized fact that occasionally similar clinical and pathological pictures may be produced by two or more different viruses. Therefore, it could not be assumed that our virus, the Traub virus, and the Armstrong-Lillie virus are identical because of a similarity in their attributes. Consequently, it was necessary to investigate the immunological relationship of the three viruses. With the cooperation of Dr. Traub and Dr. Armstrong the following cross neutralization and cross reinoculation experiments were performed.

Demonstration of Similar Protective Antibodies in the Serum of Animals Respectively Immune to Each of the Three Viruses

The cross neutralization tests were performed independently by Traub and ourselves. The method used by Traub was similar to ours as already described (1) in connection with the investigations of the neutralizing power of the serum of our two patients. In both instances the serum-virus mixtures were tested in guinea pigs. From Traub's results quoted by Armstrong and Dickens (6) and shown in Table III, it can be seen that the serum from our immune monkey and from Armstrong's immune monkey neutralized 100 lethal doses of the Traub virus which had a titer of 10^{-3}. It is equally evident from Table IV, in which our results are shown, that serum from Traub's immune guinea pig, Armstrong's immune monkey, and one of our patients (W.E.) during convalescence neutralized 100 infective doses of our virus which also had a titer of 10^{-3}.

Demonstration That Animals Immune to One Virus Resist Infection with Either of the Others

The results of the experiments summarized in Table V were obtained independently by Armstrong, Traub, and ourselves. Armstrong and Dickens (6) showed that mice immune to the Rivers-Scott virus were also resistant to the Armstrong-Lillie active agent, whereas normal mice, both from The Rockefeller Institute, New York, and the National Institute of Health, Washington, were susceptible. Traub demonstrated that mice and a guinea pig immune to the Rivers-Scott virus were equally resistant to his agent, while stock guinea pigs and stock mice from New York were susceptible. We showed that guinea pigs immune to the Traub virus were also immune to the Rivers-Scott agent, whereas stock pigs were susceptible.

When Traub made his cross reinoculation experiment he also included mice from the Princeton infected stock and found that 50 per cent of them were immune (Table V). The finding of such a high percentage of immune mice in the Princeton stock and the absence of immune animals from the stock used by us whether derived from The Rockefeller Institute, New York, or from an outside dealer (Freed) is additional evidence that the mice with which we are working are free from the virus.

TABLE III

Results of Neutralization Experiment Showing Immunological Identity of the Armstrong-Lillie, Traub, and Rivers-Scott Viruses

Dilution of virus	Serum of monkey immune to Rivers-Scott virus plus Traub virus	Serum of monkey immune to Armstrong-Lillie virus plus Traub virus	Serum of monkey immune to louping ill plus Traub virus	Serum of normal human being plus Traub virus
10^{-2}	No fever, survived	No fever, survived	Typical illness, died	Typical illness, died
10^{-3}	" " "	" " "	" " "	" " "
10^{-4}	" " "	" " "	No fever, survived	No fever, survived

The neutralization experiment was conducted by Traub in the manner described in the previous paper (1) except that the foot pad was the site chosen for inoculation.

TABLE IV

Results of Neutralization Experiment Showing Immunological Identity of the Armstrong-Lillie, Traub, and Rivers-Scott Viruses

Dilution of virus	Serum of human being (W.E.) immune to Rivers-Scott virus plus Rivers-Scott virus	Serum of normal human being plus Rivers-Scott virus	Serum of guinea pig immune to Traub virus plus Rivers-Scott virus	Serum of monkey immune to Armstrong-Lillie virus plus Rivers-Scott virus
10^{-1}	No fever, survived	Typical illness, died	No fever, survived	No fever, survived
10^{-2}	Transient fever, survived	" " "	" " "	" " "
10^{-3}	No fever, survived	Typical illness, survived	" " "	" " "

The neutralization tests were conducted in the manner described in the previous paper (1).

TABLE V

Results of Reinoculation Experiments Showing Immunological Identity of the Armstrong-Lillie, Traub, and Rivers-Scott Viruses

Animals immune to Rivers-Scott virus					Animals immune to Traub virus	
Reinoculations with Armstrong-Lillie virus*		Reinoculations with Traub virus†			Reinoculations with Rivers-Scott virus‡	
Type of animal	Result	Type of animal	Result		Type of animal	Result
	Dead		Sick	Dead		
12 mice immune to Rivers-Scott virus	0/12	10 mice immune to Rivers-Scott virus	0/10	0/10	2 guinea pigs immune to Traub virus	No fever, survived
12 normal mice Rockefeller Inst., N. Y., healthy stock	12/12	12 normal mice Rockefeller Inst., N. Y., healthy stock	12/12	11/12	2 normal stock guinea pigs	Typical illness, died in 13 days
6 normal mice Nat'l Inst. of Health, Wash., healthy stock	5/6	12 normal mice outside dealer (Freed), healthy stock	12/12	9/12		
		12 mice, Rockefeller Inst. at Princeton, infected stock	6/12	6/12		
		Guinea pig immune to Rivers-Scott virus	No fever, survived			
		2 normal stock guinea pigs	Typical illness, died in 12 days			

The results of the experiments in which mice were used have been expressed as fractions, the denominators representing the number of mice inoculated, the numerators the number of mice either sick or dead.

The inoculations were made intracerebrally in all animals.

* Tests were performed by Dr. Armstrong.

† Tests were performed by Dr. Traub.

‡ Tests were performed by Drs. Rivers and Scott.

A comparison of the reactions of laboratory animals to our virus, the Traub virus, and the Armstrong-Lillie virus, and the results of cross neutralization and cross reinoculation experiments clearly indicate that the three viruses are closely related if not identical.

DISCUSSION

The immunological identity of the 3 strains of virus independently discovered permits us to use data already published by each of the other workers concerning the different strains in attempts to determine the extent of the distribution of the virus among laboratory animals and its importance as a cause of disease in human beings.

The virus under consideration was isolated in 3 different laboratories from 3 different hosts. Armstrong and Lillie (5) recovered the active agent from the brain of a *Macacus rhesus* monkey well along in a series of monkeys originally inoculated with material from the brain of a person who had died of the St. Louis type of encephalitis. Later Armstrong and Wooley (7) found evidence of immunity to the virus in some of their stock monkeys and even isolated it from the brain of an animal dying of experimental poliomyelitis. Traub (4) discovered his strain of this virus in albino Swiss mice and at first thought them to be the natural host. We have definitely demonstrated that our strain of the virus was obtained from the spinal fluid of human beings in whom it caused a meningitis.

Sufficient evidence is not yet available to estimate the ease with which infection can spread from animal to animal within a given species, or from one species to another. The fact that the malady has persisted for quite a while in stock mice at Princeton, certainly indicates that the virus can spread spontaneously from mouse to mouse. But, judging from the results of our experiments detailed above, its contagiousness is not very great. Armstrong did not encounter immune monkeys at the beginning of experiments on this virus, but at a later period he did. This may have been due to coincidence, but was more probably consequent on a spontaneous spread of the disease, once established, among the stock monkeys.

In regard to the spread of the disease, caused by the new virus, from lower animals to man or *vice versa*, the evidence is limited, but that which is available seems to indicate that it is more likely to go from man to lower animals than in the opposite direction.

None of the investigators actively engaged in work with the virus has become sick. Armstrong and Wooley (7) have reported that the serum of four workers constantly in contact with infected monkeys contains no neutralizing antibodies; Traub (8) states that, after working at least 2 or 3 months with the virus, two investigators had no neutralizing antibodies in their serum; and we have found that an individual in contact with the virus for 9 months still possesses no protective antibodies. Finally, Traub has allowed us to see evidence, which he will publish later, in favor of the idea that susceptible animals may contract the disease from a human carrier.

The evidence regarding the source of the infection of our patients is not conclusive. In the case of W.E. we can state definitely that it is unlikely that he came in contact with the virus from an animal source, because, so far as is known, there was no infected stock in the Institute where he was working at the time he became ill. R.E.S., however, worked with a stock of mice, which was later found to be infected, for 3 months prior to the onset of his illness, and therefore may have contracted the disease in that manner. Nevertheless as stated above, the disease does not readily spread to man from animals.

It seems that a source of virus in lower animals, if one exists, would be of little menace to man. Thus one is left with the probability that the source of infection for man is a human one. Evidence in favor of such an idea is difficult to obtain, yet that which is available does not nullify it and consists in finding either the virus or neutralizing antibodies against it in human beings geographically scattered who do not present histories of previous contact with infected lower animals.

We undoubtedly obtained the virus from the spinal fluids of two patients, one of whom gave no history of contact with infected animals. Armstrong and Lillie's (5) original strain C.G. may have come from the brain of a human being, because, at the time of its isolation, there was no evidence of a latent infection with the virus in their stock monkeys. Unfortunately, it is far from being clear that their second strain A.O. came from man, because, in the same paper (7) in which its isolation was described, Armstrong refers to the discovery of another strain in a monkey dying of experimental poliomyelitis.

The evidence regarding the presence of neutralizing antibodies in the serum of individuals geographically scattered is more striking. Armstrong and his colleagues (6, 7) have found protective antibodies

in the serum of 6 people living in different parts of the country from California to the District of Columbia. 4 of them gave histories of a previous illness similar to that observed in our patients, 1 had recovered from the St. Louis type of encephalitis and also possessed antibodies against the virus of that malady, and 1 had no history of any recent previous illness except grippe. Traub (8) found antiviral substances in the serum of 2 of the men working in the animal house at Princeton. It is true that 3 of the 8 individuals with antibodies in their serum were located in the laboratories at Princeton or Washington, yet the others were so geographically scattered that one necessarily considers the possibility of an endemic focus of the virus in human beings.

The clinical picture of the disease definitely known to be caused by the virus, that in our two patients and in the one cited by Armstrong and Dickens (6), is seen not infrequently, and in 1925 Wallgren (9) suggested that it probably was characteristic of a clinical entity. For such an entity he adopted the name "acute aseptic meningitis," and laid down the following criteria for its diagnosis. Sudden onset of signs of meningitis associated with a lymphocytic pleocytosis in a spinal fluid free from bacteria, a short benign course, absence of a focus of infection in the neighborhood of the brain, *e.g.*, sinusitis, and absence from the community of diseases known to be capable of producing a meningeal irritation.

The problem would be considerably simplified if the discovery of the new virus provided an etiological agent for Wallgren's clinical entity. Facts, however, indicate that the virus is the cause of only a few cases diagnosed as acute aseptic meningitis. We (1) have failed to find the active agent in the spinal fluid of 7 patients suffering from an illness that satisfied Wallgren's criteria[2] and 3 more individuals with a lymphocytic meningeal reaction concerning which sufficient details are not available to determine whether it definitely met the requirements. Furthermore, we have been unable to demonstrate neutralizing antibodies against our virus in the serum col-

[2] The figures include the results of studies of an epidemic of acute aseptic meningitis in Philadelphia during July and August, 1935. The privilege of investigating these cases was afforded by Stokes (10).

lected from 17 patients recovered from diseases diagnosed as acute aseptic meningitis,[2] and from 5 more individuals convalescent from a lymphocytic meningitis of unknown origin.

It has already been indicated that our virus produces for the most part a reaction in the meninges. Many viruses that attack the central nervous system also cause some reaction in the meninges, but, in addition to this type of damage, these active agents tend to injure some particular part of the brain or cord with a resultant set of symptoms and signs held to be more or less characteristic of certain maladies, *e.g.*, the anterior horn cells are especially picked out by the virus of poliomyelitis, while the nuclei of cranial nerves are likely to be damaged in Economo's disease. In regard to the new virus, however, there is no evidence from the meager clinical data or from the more extensive experimental material that any part of the nervous system, except the meninges and choroid plexus, is especially involved. Hence signs of a meningitis are the sole clinical manifestation of its activity.

Signs of a meningitis may be the only clinical manifestation of abortive attacks of poliomyelitis, of the St. Louis type of encephalitis, or of epidemic encephalitis in its meningeal form. Moreover, meningeal signs may occasionally be the outstanding feature of other virus diseases, such as herpes zoster and mumps, in which their usual manifestations are absent or poorly developed. From what has been said, it is obvious that incorrect diagnoses are likely to occur not infrequently until a more general effort is made to determine the etiological agent functioning in each instance.

SUMMARY

Evidence was presented substantiating the idea that our active agent is a virus. The reactions produced in laboratory animals by the active agent were described, and a comparison of it with other viruses was made. The results of experiments indicating the immunological identity of our virus, Armstrong and Lillie's virus of lymphocytic choriomeningitis, and Traub's virus were given in detail. Finally, the evidence in regard to its natural host and its importance as a factor in diseases of human beings was presented and discussed.

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EXPLANATION OF PLATES

PLATE 30

FIG. 1. Section of brain from an infected mouse showing cellular infiltration of the meninges and absence of changes in the brain substance. Hematoxylin and eosin. × 40.

FIG. 2. Higher magnification of the meninges of an infected mouse showing that the exudate consists chiefly of mononuclear cells. Hematoxylin and eosin. × 250.

FIG. 3. Section of lung from an infected guinea pig showing pneumonic consolidation. Hematoxylin and eosin. × 40.

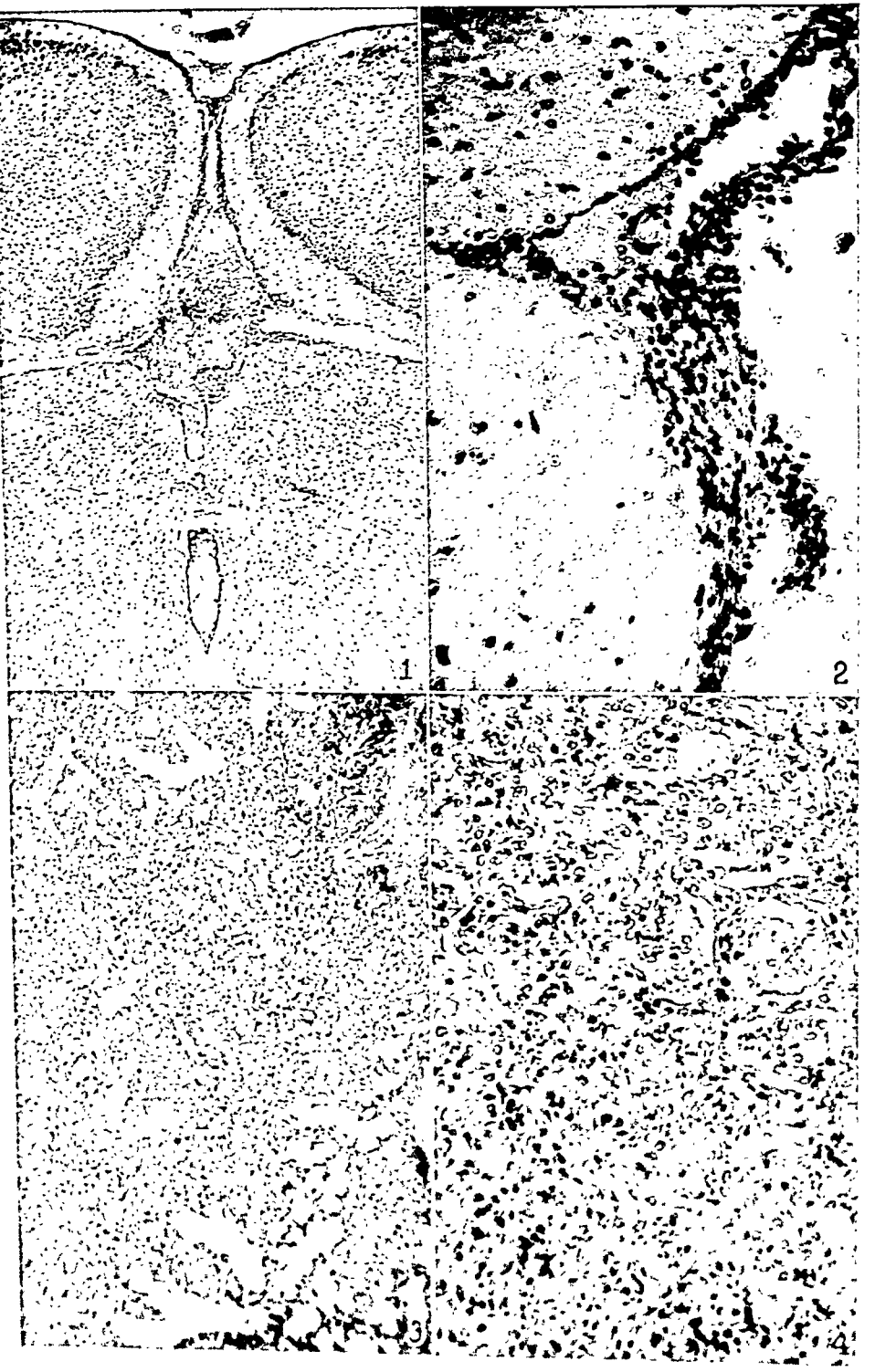
FIG. 4. Higher magnification of section shown in Fig. 3 illustrating the interstitial character of the pneumonia. Hematoxylin and eosin. × 250.

PLATE 31

FIG. 5. Section of choroid plexus of an infected monkey. The arrows indicate the portions shown under higher magnification in the next two figures. Hematoxylin and eosin. × 40.

FIG. 6. Higher magnification of section shown in Fig. 5 illustrating the dense mononuclear infiltration around a blood vessel in the choroid plexus as contrasted with the comparative absence of infiltration surrounding a blood vessel of similar size in the subependymal tissues, which also show a mild diffuse inflammatory reaction. Hematoxylin and eosin. × 250.

FIG. 7. Higher magnification of another portion of section shown in Fig. 5 illustrating the distortion of the cuboidal ependymal cells by the perivascular inflammatory exudate. Hematoxylin and eosin. × 250.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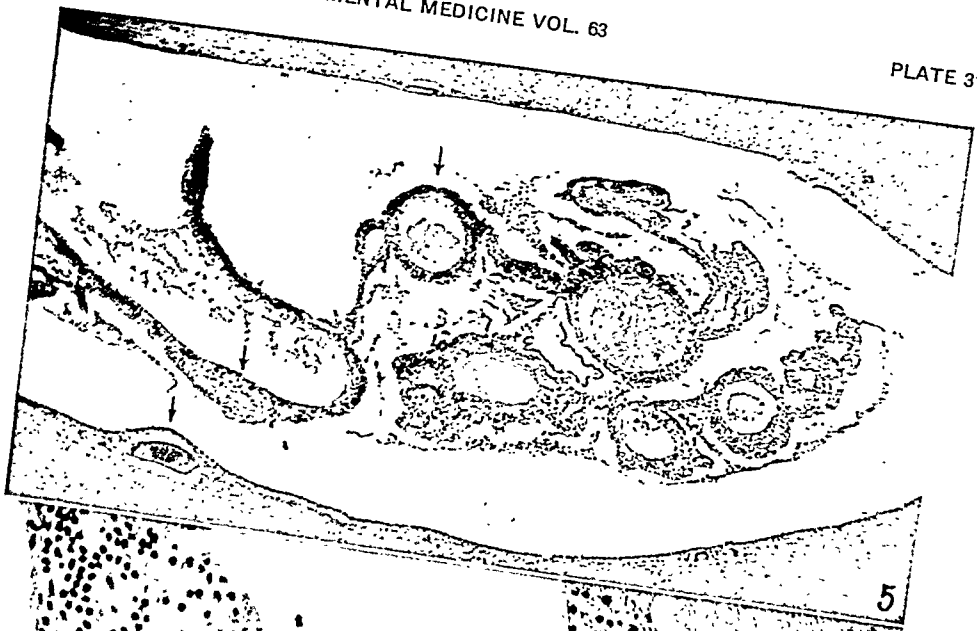

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THE LIMITED NEUROTROPIC CHARACTER OF THE ENCEPHALITIS VIRUS (ST. LOUIS TYPE) IN SUSCEPTIBLE MICE

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Plates 32 to 34

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The finding of a small experimental animal, the mouse, susceptible to the St. Louis encephalitis virus (1, 2) has provided a means of studying quantitatively a human type of central nervous system disease. The highly susceptible mice we have used are uniform in their reactions to the virus and following nasal instillation, regularly contract encephalitis. Preliminary tests on the infectivity and distribution of virus injected by different routes have been reported (3). The present paper describes in detail the limited neurotropism of the virus, its invasiveness following nasal instillation, its transmission from nose to brain by the olfactory route, and its establishment in the brain several days prior to the onset of clinical disease.

Predilection of Virus for Nervous Tissue (Neurotropism)

Results of Injecting Virus Intracerebrally.—Experiment 1. Jan. 22, 1934.—Twenty-five Swiss mice were injected intracerebrally with 0.03 cc. of mouse brain virus, Strain 3, diluted 1 to 50 in hormone broth. Thereafter, at intervals from 10 minutes to 4 days following injection, thirteen mice were tested for content of virus in brain, blood, and spleen, and twelve were reserved as controls. Mice to be tested were etherized, bled from the heart, and their brains and spleens removed and emulsified with alundum. The materials were then prepared in serial tenfold dilutions and 0.03 cc. of each was injected intracerebrally into two Swiss mice. The titre of virus in test material was taken as the highest dilution killing at least one of the two injected mice. This dilution was then expressed as numbers of intracerebral lethal doses. For example, the brain of a Swiss mouse dying of experimental encephalitis is fatal when injected into mice in doses of 0.03 cc. of a 10^{-7} dilution, which is 3×10^{-9}, or roughly 10^{-8}. Hence the brain content of virus is said to be 10^{8} intracerebral lethal doses for susceptible mice.

The results of this experiment are given in Table I. The original inoculum of about 10^7 intracerebral lethal doses was fatal to the controls on the 3rd and 4th days. Brain content of virus 10 minutes after injection was 10^6 and blood content at the same time, 10^4 doses. At 5 hours, the brain titre dropped to 10^3 doses and then rose rapidly to a maximum not measured in this test. Blood became non-infective

TABLE I

Brain, Blood, and Spleen Content of Encephalitis Virus in Susceptible Mice Following Intracerebral Injection of 10^7 Lethal Doses

Mouse No.	Time interval injection to test	Content of virus, 0.03 cc. of each dilution to two mice								
		Brain					Blood			Spleen
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Undiluted	10^{-1}	10^{-2}	10^{-1}
1	10 min.	5,* 5	5, 5	6, 7	11		5, 6	5, 5	8, 8	N.T.
2	20 "	5, 5	5, 6	6, 7		7	4, 4	5, 6	7, 9	"
3	1 hr.	D, 6	6, 8	8	6		5, 5	5, 7		"
4	3 hrs.	5, 6	6, 7	7, 12			5, 7			"
5	5 "	D, 6								"
6	7 "	6, 7		6						7
7	9½ "	5, 5	6, 8	7, 9						
8	10 "	6, 6	5, 5					N.T.	N.T.	
9	1 day	4, 6	5, 6	5, 5	5, 9	8		"	"	5, 5
10	2 days	D, 4	4, 4	4, 4	5, 5	7, 7	D, 7	"	"	5, 7
11	2 "	4, 4	4, 4	4, 4	4, 5	5, 6	6, 7	"	"	7, 7
12	3 "	4, 4	4, 5	5, 6	6, 6	5, 5	6	"	"	
13	3 "	4, 5	4, 6	6, 6	6, 7	5, 7	7	"	"	

* = duration of life of mouse in days.
Blank spaces = mice remained well 21 days.
D = mouse died immediately following injections.
N.T. = dilution not tested.

at 5 hours but at 2 and 3 days contained virus in 10^2 titre. Spleens contained virus in accordance with expectation from the blood findings.

Tests were made on susceptible mice injected with smaller amounts of virus, 10^5 and 10^3 lethal doses, with similar results. The brain titre of virus increased after a lag to 10^8 or 10^9, while the blood was positive only immediately following injection and preceding death. Brain and cords of these mice showed characteristic lesions; other organs appeared normal (2). Apparently, therefore, virus injected

intracerebrally into susceptible mice exhibits a predilection for nervous tissue.

Results of Injecting Virus Intraperitoneally or Subcutaneously.—Virus injected intraperitoneally or subcutaneously rapidly invaded the blood stream and survived in the spleen and yet did not harm the animal unless overwhelming doses were used or the brain was injured.

TABLE II

Brain, Blood, and Spleen Content of Encephalitis Virus in Susceptible Mice Following Intraperitoneal Injection of 10^7 Intracerebral Lethal Doses

Mouse No.	Time interval injection to test	Content of virus. 0.03 cc. of each dilution to two mice					
		Blood			Brain		Spleen
		Undiluted	10^{-1}	10^{-2}	10^{-1}	10^{-2}	10^{-1}
1	10 min.	4,* 4	5, 6	6, 6	5		
2	20 "	4, 4	4, 5	6, 6	7		
3	1 hr.	5, 5	4, 6	7, 7	6, 8		
4	3 hrs.	4, 5	6, 6	6, 8			
5	5 "	7, 7					
6	7 "	10					5, 6
7	9 "						6, 8
8	1 day		N.T.	N.T.		N.T.	5, 5
9	1 "		"	"		"	5, 7
10	2 days	5, 5	"	"		"	6, 7
11	2 "	8	"	"		"	6, 6
12	3 "		"	"		"	5, 5
13	3 "	6, 8	"	"	7, 8	"	5, 6
14	4 "	6, 7	"	"		"	5, 6
15	4 "		"	"		"	6, 6
16	5 "	8	"	"	8	"	
17	5 "		"	"		"	
18	15 "						5
19	15 "						

* = duration of life of mouse in days.
Blank spaces = mice remained well 21 days.
D = mouse died immediately following injections.
N.T. = dilution not tested.

Experiment 2. Jan. 29, 1934.—Thirty Swiss mice were injected intraperitoneally with 0.5 cc. of virus diluted 1 to 50. At intervals thereafter, from 10 minutes to 15 days, nineteen mice were sacrificed and examined for content of virus in blood, brain, and spleen, and eleven were reserved as controls. Materials for testing were obtained, prepared, and injected as described in Experiment 1.

The results of this experiment are summarized in Table II. The original dose injected intraperitoneally per mouse was equivalent to 10^7 intracerebral lethal doses. One of the eleven controls died of encephalitis on the 6th day following the injection; the others, together with the nineteen test animals, remained well. The first animal was sacrificed 10 minutes after injection and virus was found in its blood in large amounts, 10^4 titre. Similar quantities were present in the blood of mice at 20 minutes, 1, and 3 hours. At 5 and 7 hours, and irregularly thereafter, the undiluted blood contained virus. The titre of virus in the brain 10, 20, and 60 minutes following injection was 10^3 but negative thereafter, save in single mice, on the 3rd and 5th days respectively. Virus was present in the spleen in 10^3 quantities when first tested 7 hours after injection and regularly through the 4th day, and in one mouse on the 15th day.

Tests with different doses of virus and different strains of susceptible mice gave essentially the same results except that if the injected dose was smaller or the strain of mice employed was slightly more resistant, the resulting titres of virus in blood and brain were correspondingly less. Virus content of liver, lung, and kidney was found to be negligible. The spleen, however, contained virus over periods as long as 30 days. Similar results were obtained when the virus was injected subcutaneously.

According to these experiments, virus circulating in the blood vessels of the brains of intraperitoneally injected mice is relatively incapable of causing encephalitis and yet when injected directly into brain tissue through the dura is extremely pathogenic (Experiment 1). It appears likely, therefore, that the conditioning factor is trauma of the brain. This supposition was tested by injecting mice intraperitoneally with virus and following it by a subdural injection of sterile starch, according to the method of the yellow fever protection test (4).

Experiment 3. Mar. 12, 1935.—Dilutions of virus from 10^{-2} to 10^{-6} were each injected intraperitoneally in 0.5 cc. quantities into ten Swiss mice. After 60 minutes, five of the mice given each dilution of virus received an intracerebral injection of 0.03 cc. of a 2 per cent sterile starch solution and the remainder served as controls. At the same time the intracerebral virulence of the virus suspension was determined.

The results of the test are shown in Table III. The intracerebral titre of the virus was as usual, 3×10^9. Mice receiving intraperitoneally the largest dose of virus, 0.5 cc. of 10^{-2} dilution, 10^7 intracerebral lethal doses, died of encephalitis regardless of the presence or absence of cerebral trauma. Of those receiving the 10^{-3} and 10^{-4} dilution of virus, 10^6 and 10^5 intracerebral lethal doses, only animals subsequently traumatized developed encephalitis and died. One of

TABLE III

Effect of Intracerebral Trauma on the Pathogenicity of Encephalitis Virus Injected Intraperitoneally in Susceptible Mice

Five mice injected intraperitoneally 0.5 cc. in dilutions	Presence of trauma	Duration of life	Mortality
		days	*per cent*
10^{-2}	+	5, 5, 6, 6, 6	100
10^{-2}	0	5, 5, 6, 6, 11	100
10^{-3}	+	5, 6, 6, 6, 6	100
10^{-3}	0	Remained well	0
10^{-4}	+	6, 6, 6, 7, 12	100
10^{-4}	0	Remained well	0
10^{-5}	+	6	20
10^{-5}	0	Remained well	0
10^{-6}	+	" "	0
10^{-6}	0	" "	0

Intracerebral virulence of virus

	0.03 cc. subdurally in dilutions			
	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Duration of life, *days*	4, 4, 4, 4	4, 4, 4, 5, 5, 6	5, 5, 6, 6, 6, 7	6, 7, 7, 8
Mortality rate, *per cent*	100	100	100	100

five mice receiving the 10^{-5} dilution of virus plus trauma died; the remainder, together with the five controls, were unharmed. All mice given the 10^{-6} dilution, 10^1 intracerebral lethal doses, remained well. The experiment shows that virus in the blood stream not ordinarily infectious for the intact brain becomes so when the brain is traumatized.

Results of Instilling Virus into the Nose.—A number of neurotropic encephalitis-producing viruses are infectious for mice by the nasal

route,—for example, louping ill, yellow fever, and equine encephalomyelitis. It was not surprising to learn, therefore, that the St. Louis virus was similar in this respect. Its ready penetration from nose to brain and its rapid multiplication in the brain are illustrated in the following experiment.

TABLE IV

Brain and Spleen Content of Encephalitis Virus in Susceptible Mice Following Nasal Instillation of 10^5 Intracerebral Lethal Doses

Mouse No.	Time interval injection to test	Content of virus. 0.03 cc. of each dilution to two mice							
		Brain							Spleen
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-1}
	days								
1	1			N.T.	N.T.	N.T.	N.T.	N.T.	
2	1			"	"	"	"	"	
3	2	5,* 5	6, 6	11	"	"	"	"	7, 7
4	2	5, 5	6, 10		"	"	"	"	5, 7
5	3	5, 5	6, 6	D, 6	9	"	"	"	6, 6
6	3	6, 6	5, 6	8, 9				"	6, 7
7	4	N.T.	5, 5	5, 5	5, 5	7, 7	8	"	5, 5
8	4	"	5, 7	8, 8				"	
9	5	"	4, 6	4, 6	6, 6	6, 6	6, 6	"	5, 6
10	5	"	4, 6	4, 6	6, 6	6, 6	6, 6	"	5, 7
11	6	"	N.T.	5, 5	5, 6	5, 5	5, 6	6, 6	5
12	6	"	"	5, 5	5, 5	5, 6	5, 5	6, 6	6, 6
13	7	"	"	5, 5	5, 5	5, 5	6, 6	6	5, 6
14	7	"	"	5, 7	8, 8	8, 8			8, 8

* = duration of life of mouse in days.
Blank spaces = mice remained well 21 days.
D = mouse died immediately following injections.
N.T. = dilution not tested.

Experiment 4. Dec. 28, 1934.—Brain virus was prepared and diluted 1 to 100 in the routine manner. 0.03 cc. was then dropped through a 0.25 cc. syringe with blunt needle into the nasal orifices of each of twenty Swiss mice. This quantity is equivalent to 10^7 intracerebral lethal doses and about 10^2 intranasal lethal doses. At daily intervals thereafter for 7 days, two mice were sacrificed and their brains and spleens tested for the presence of virus according to the technique described in Experiment 1. Six mice were set aside as controls. The results of the experiment are given in Table IV.

The controls died of encephalitis on the 7th and 8th days. On the 1st day after nasal instillation, brains of the two test mice showed no virus. On the 2nd day, however, the brain titres of virus in the two tested mice were 10^5 and 10^4 respectively. On the 3rd day the brain titres were 10^6 and 10^5, on the 4th day, 10^8 and 10^5, on the 5th day, 10^8, 6th day, 10^9, and 7th day, 10^9 and 10^7. The spleens contained virus on the 2nd day and consistently thereafter.

Five additional experiments of the same sort were made demonstrating the presence of virus in the brain 48 hours after nasal inoculation, its rapid multiplication there to a titre of 10^9 in 6 days, and furthermore its presence in the spleen after 48 hours. The experiments also included nineteen futile attempts to recover virus from the entire emulsified brain 5 minutes to 24 hours after nasal instillation of virus. In addition, unsuccessful searches for virus in the undiluted blood were made on twenty-seven mice 5 minutes to 6 days after inoculation. Seventeen bleedings were made on seven mice within 19 minutes and on seventeen additional mice within 90 minutes of inoculation. In two or three instances the blood killed one of two injected mice after prolonged incubation periods or protected mice against subsequent intracerebral injection. Spleens, however, contained virus regularly from the 2nd to 7th days after nasal instillation of virus.

The above experiments demonstrate the predilection of the encephalitis virus for the central nervous system. Its tendency to localize, multiply, and form lesions almost exclusively in the brain and cord following intracerebral or intranasal injections, together with its inability, when injected directly or indirectly into the blood stream, to gain a foothold there in the absence of cerebral trauma, bespeaks its neurotropism. And yet the virus does find its way to the spleen following intranasal instillation, presumably *via* the blood, and survives there a surprisingly long period of time. So marked was this property that experiments were made to determine whether the virus could actually multiply in the spleen.

Experiment 5. Mar. 4, 1935.—Mouse brain virus was prepared, diluted 1 to 10, and injected in 0.5 cc. quantities intraperitoneally into three Swiss mice. 24 hours later the mice were sacrificed, spleens removed, emulsified, and taken up in about 1.8 cc. of broth. 0.5 cc. of this spleen emulsion was then injected intraperitoneally into each of three mice and 0.03 cc. intracerebrally into two mice.

The intraperitoneally injected mice were sacrificed at 24 hours and their spleens removed and prepared as above. Again the spleen emulsion was injected intraperitoneally into three mice. This procedure was repeated forty-four times at intervals of 24 to 48 hours with accompanying intracerebral injections into two mice after the 1st, 2nd, 5th, 10th, 16th, 21st, 24th, 27th, 32nd, 38th, and 44th passages to determine whether the spleen emulsions still contained active virus. The 44th passage was made Apr. 29, 1935, 56 days after the 1st passage.

The virus remained active after 44 spleen passages in this test and after 32 passages in a similar experiment. All spleen emulsions tested intracerebrally in mice gave rise to encephalitis fatal on the 6th to 8th days. The initial intraperitoneal inoculum of 10^8 intracerebral doses was probably diluted by each passage due to failure of spleens to take up all injected virus and to the difficulty of reinjecting the entire amounts of prepared spleen emulsion. It seems likely, therefore, that not only prolonged survival but actual multiplication of virus took place in the spleens of susceptible mice.

Infective Route of Virus from Nose to Brain

By what route does virus instilled into the noses of susceptible mice travel to the brain and set up a fatal encephalitis? Not by the blood stream, though minimal amounts probably reach the circulation from the nasal mucosa. For it has been shown that virus in the blood does not readily induce encephalitis in the absence of brain injury. A more likely possibility is that virus extends directly from nasal mucosa to brain by way of the olfactory tract, either axis cylinders or perineural spaces.

If the direct extension idea is correct, one would find virus and lesions following intranasal instillation, not scattered irregularly throughout the brain at any given time, but first in the olfactory bulbs and later in the brain proper. Evidence of this orderly progression of virus and lesions is given in the following experiments.

The first tests dealt with regional distribution of virus in brain and cord.

Experiment 6. June 4, 1935.—0.03 cc. of mouse brain virus diluted 1 to 100 was dropped into the nares of twelve Swiss mice. At daily intervals thereafter mice were sacrificed and tested for the presence of virus in the olfactory bulbs and piriform area, the remainder of the brain, and the cord. The olfactory bulb and piriform tissue was obtained by placing the ventral surface of the brain uppermost

and cutting from posterior to anterior to remove the piriform area and olfactory bulbs. The remainder of the brain was tested separately. The spinal cord was tested *in toto*. The various tissues were triturated in a mortar with alundum and diluted 1 to 10 with broth. Each emulsion was then injected intracerebrally into two Swiss mice to detect the presence of active virus.

Table V gives the results of the experiment. The three controls died on the 7th and 8th days. Two mice tested 24 hours and one tested 48 hours after nasal instillation showed virus in the bulb and

TABLE V

Content of Encephalitis Virus in Central Nervous System Following Nasal Instillation of 10^5 Lethal Doses

Mouse No.	Time interval injection to test	Presence of virus. 0.03 cc. of dilution 10^{-1} to two mice		
		Olfactory area*	Remainder of brain	Cord
	days			
1	1	6**		
2	1	6, 6		
3	2	7, 8		
4	2	4, 5	5	
5	3	5, 7	5, 5	
6	3	5, 5	5, 6	
7	4	5, 6	4, 5	6, 6
8	5	4, 4	4, 6	6, 6
9	7	N.T.	4, 5	5, 5

* Ventral portion of piriform lobes plus olfactory bulbs.

** = duration of life of mouse in days.

Blank spaces = mice remained well 21 days.

D = mouse died immediately following injections.

N.T. = dilution not tested.

piriform tissue but none in the remainder of the brain or in the cord. The second mouse tested at 48 hours, and the two tested on the 3rd day contained virus in bulbs and piriform tissue and in the remainder of the brain but not in the cord. On the 4th, 5th, and 7th days, virus was found in the three regions tested, olfactory bulb and piriform area, remainder of the brain, and the cord.

Experiment 7. June 20, 1935.—A similar test was made with tissue from olfactory bulbs alone, piriform area, and remainder of the brain. The olfactory bulbs were severed close to the brain and the piriform areas removed as in the

previous experiment. A total of ten mice were sacrificed and tested at 4, 5, 6, 24, 25, 28, 30, and 48 hours. Four additional mice were reserved as controls.

The results of this experiment are summarized in Table VI. The four controls died between the 7th and 9th days Virus was not found until 25 hours after instillation, when it was present only in the olfactory bulbs. At 28 hours it was present in olfactory bulbs and piriform area; in another mouse, at 30 hours, only in the bulbs. Likewise in one mouse at 48 hours, virus was found both in the bulbs and

TABLE VI

Content of Encephalitis Virus in Olfactory Bulbs and Brain Following Nasal Instillation of 10^5 Lethal Doses

Mouse No.	Time interval injection to test	Presence of virus. 0.03 cc. of dilution 10^{-1} to two mice		
		Olfactory bulbs	Piriform area	Remainder of brain
	hrs.			
1	4			
2	5			
3	6			
4	6			
5	24			
6	25	7*		
7	28	6, 7	7	
8	30	6, 7		
9	48	4, 5	6, 7	
10	48	5, 5		

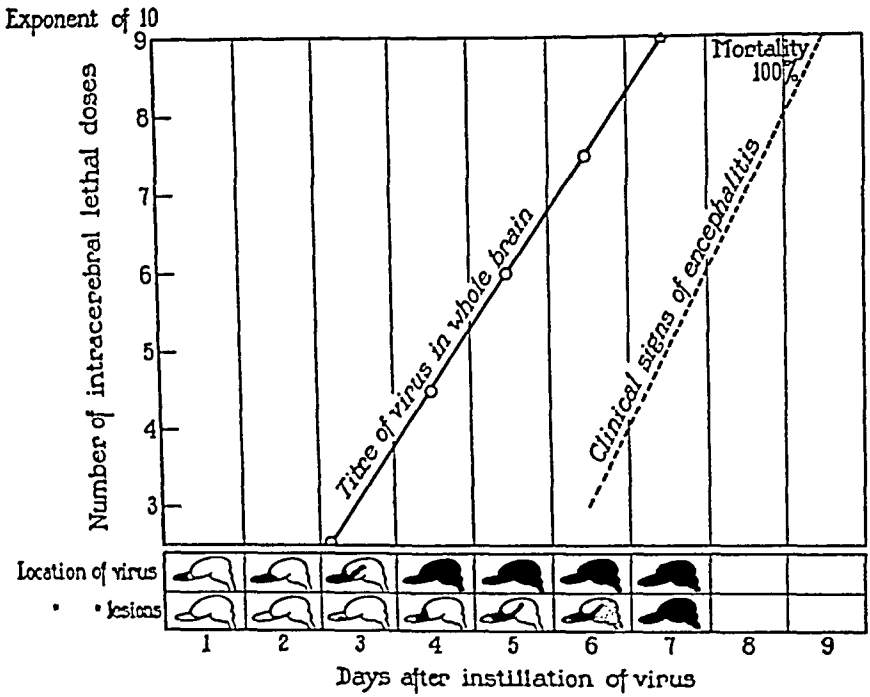
* = duration of life of mouse in days.
Blank spaces = mice remained well 21 days.
D = mouse died immediately following injections.
N.T. = dilution not tested.

piriform area; in the other, however, only in the bulbs. The remainder of the brain contained no virus for 48 hours following nasal instillation.

Taken together, the tests show that virus is demonstrable at 24 hours in the olfactory bulbs but not in the brain and the cord, that at 48 hours it is abundant in the bulbs and present occasionally in the anterior piriform area of the brain. At 3 days, however, it is present in the remainder of the brain, and at 4 days has reached the spinal cord.

The route by which virus travels from nose to brain was studied

further by histological methods. Mice given virus intranasally were sacrificed in batches at daily intervals thereafter and their olfactory bulbs, brains, and cords sectioned and stained to determine the time, location, and nature of the initial lesion and its subsequent advance.



TEXT-FIG. 1. Time relation between onset of encephalitis in the nasally infected susceptible mouse and the appearance of tissue alterations and virus in the brain.

Two lots of mice were studied. The first consisted of thirty-two Swiss mice which received on Dec. 16, 1933, 0.03 cc. of a 10^{-2} brain virus suspension intranasally. Daily for 7 days following the instillation, four mice were sacrificed, their brains and cords removed, fixed in Zenker's acetic solution, and sectioned. The four remaining mice died of encephalitis on the 8th and 9th days. The second lot of sixteen mice given virus in June, 1935, was treated similarly. Sections of brain were taken at the levels of the lateral ventricles, Ammon's horn, and midcerebellum. The cord was examined at the cervical, thoracic, and lumbar levels. Furthermore, serial sections were made of the olfactory bulbs and portions of the brain anterior to the lateral ventricles. Ten consecutive sections each of 5 $\mu\mu$

thickness were alternately prepared and discarded to furnish more than 100 sections of the anterior olfactory region from each mouse. The tissue was stained by Giemsa's method and with eosin-methylene blue.

24 Hours after Nasal Instillation.—The virus is present in the olfactory bulbs (Text-fig. 1). No lesions were recognized, however, in the serial sections of olfactory bulbs and brains of the five mice examined at this time.

2 Days after Nasal Instillation.—The virus is abundant in the olfactory bulbs and is present in the piriform area of the brain proper. Again, serial sections of olfactory bulbs and brains of the five mice studied at this time appeared normal.

3 Days after Nasal Instillation.—The virus has reached a titre in the whole brain of 10^3 lethal doses. Of the five mice examined at this time, two appeared normal and three showed a definite and similar lesion. On the ventral aspect of the olfactory bulbs near the brain, one or two focal accumulations of round cells and an occasional polymorphonuclear leucocyte were noted in the Virchow-Robin spaces about the blood vessels in or beneath the pia (Fig. 1). In addition, the neighboring superficial blood vessels were congested and a few leucocytes were scattered about the bundles of non-medullated nerves.

4 Days after Nasal Instillation.—The virus content of the whole brain titres 10^4.

All five mice examined showed lesions similar in character but varying slightly in extent. The congestion of superficial blood vessels and perivascular exudate were more advanced than on the 3rd day, extending throughout the ventral posterior two-thirds of the olfactory bulbs. In one mouse the lesion had reached the ventral, medial regions of the piriform area (Figs. 2, 3). Likewise the scattered leucocytes about the olfactory nerve bundles were more conspicuous. In addition, a second type of lesion was noted in three mice, namely, hyperplasia of pial endothelium, extending in some instances only along the ventral posterior third of the olfactory bulbs, but in the mouse showing the most advanced changes continuing over the ventral, medial portion of the piriform lobes (Fig. 4).

5 Days after Nasal Instillation.—The virus has reached a titre in the whole brain of 10^6 lethal doses. The animals sacrificed at this date still appeared clinically healthy (Text-fig. 1). Two of four mice examined showed an extension of the exudative and hyperplastic lesions over the ventral surfaces of olfactory bulbs and piriform lobes. The remaining two mice showed an abatement of these lesions and the beginnings of the third and most conspicuous change, namely, nerve cell necrosis. First to show damage were the pyramidal nerve cells of the olfactory bulbs and piriform lobes. They were in various stages of necrosis. The cytoplasm of some was deeply eosin-staining; in others, it was eosin-staining and granular. Other cells showed a shrunken, eosin-staining cytoplasm and nucleus in various stages of pycnosis. Some cells were shrunken to a mere dot of a nucleus in a fragment of eosin-staining cytoplasm. Elsewhere the cells were entirely missing and the tissue took on a punched out appearance. Relatively little exudate was present nearby.

This nerve cell necrosis did not antedate, in so far as we could determine, the inflammatory lesion. Early nerve cell changes were searched for in the 3 day and

4 day material by the use of Goodpasture's carbol-anilin-fuchsin stain with no evidence of chromatolysis or alteration of Nissl substance. Glial cells were examined, isolated nerve cells, and occasional nerve cells in an inflamed area on the 3rd day, but the impression remained that inflammation preceded specific, obvious nerve cell involvement by 24 to 48 hours.

6 to 8 Days after Nasal Instillation.—The brain titre of virus reaches a maximum of 10^9 lethal doses. The animals all develop encephalitis and die. The exudative lesion was found throughout the brain and cord. Nerve cell necrosis was extensive and in each animal appeared to develop progressively along the olfactory tracts. In all cases, the pyramidal cells of the olfactory bulbs and piriform lobe were first affected and here the inflammatory and hyperplastic lesions were subsiding. In some the olfactory ganglion and tubercle were involved. In others the necrosis had extended to the central grey substance, anterior limbic area, and hypothalamus. Most characteristic on the 6th day, however, was the extension of necrosis to the pyramidal cells of Ammon's horn (Fig. 5). These large nerve cells, together with similar ones in the piriform lobe, were invariably affected. Finally, nerve cells of the thalamic region and even certain non-olfactory areas of the cortex became involved. Scattered nerve and glial cells everywhere, both in brain and cord, became necrotic. The basal ganglia, cerebellum and motor cells of the anterior horns and the posterior root ganglion cells appeared to be spared for the most part, but by the 8th day very little of the brain remained normal (Fig. 6).

In summary (Text-fig. 1), the first lesion occurred, following nasal instillation of virus, on the 3rd day and consisted of dilatation of subpial blood vessels with exudation of round cells and polymorphonuclear leucocytes on the ventral surface of the olfactory bulbs. On the 4th day, this lesion spread to the pia covering the ventral surfaces of the piriform lobes and a second alteration was noted in these areas, namely, hyperplasia of pial endothelium. Finally, on the 4th or 5th day, necrosis of pyramidal nerve cells in the olfactory bulbs and piriform areas appeared. On the 6th and 7th days, the nerve cell necrosis spread to Ammon's horn, hypothalamus, and thalamus. Other types of nerve cells scattered through brain and cord were involved and perivascular accumulations of round cells were widespread.

DISCUSSION

The foregoing data bear on the classification and pathogenesis of the St. Louis encephalitis virus in susceptible mice. It is neurotropic according to general definition, in that it multiplies and forms lesions chiefly in the brain and attacks nerve cells directly. It invades the brain by the olfactory but not by the blood stream route. But it is not

strictly neurotropic in the sense of being nerve cell specific, in that it multiplies in the spleen.

Whether virus travels from nose to brain by nerve axons or perineural spaces is difficult to determine. Favoring nerve cell transmission is the evidence of general neurotropism of the virus, its multiplication in olfactory tissue, and its orderly progress by way of the olfactory tracts. When it comes to discovering the situation, progression, and character of central nervous system lesions following nasal instillation of virus, however, the data are less convincing, since lesions in a given region did not appear until 48 hours after virus was demonstrable. For example, the primary lesion was found in the olfactory bulbs on the 3rd day but virus, already present there 2 days previously, had extended to various regions of the brain proper. Situation and progression of lesions, therefore, cannot be taken to define precisely the site and progress of virus. The same difficulty obtains with respect to neurotropic louping ill (5) and yellow fever virus in the mouse (6) where considerable time elapses between the arrival of virus and the development of lesions. One further difficulty confronts us in testing the theory of axon transmission of this virus, namely, that inflammation was found definitely to antedate the nerve cell lesions. True, unseen alterations of nerve cells may have preceded the pouring out of leucocytes in the Virchow-Robin spaces, but the evidence at hand indicated without exception that at least 24 hours elapsed between the onset of exudation and nerve cell necrosis. On the basis of present knowledge, therefore, the question cannot be decided.

The time relation between the presence of virus and lesions in the brains of susceptible mice following nasal instillation and the onset of clinical encephalitis is noteworthy from the standpoint of therapy. 48 hours after nasal infection, 4 days before the first sign of disease, the virus is present in the brain; 2 days before disease, lesions are abundant; and finally, when the animal falls ill, virus in the brain has increased to a titre of 10 million intracerebral lethal doses. This fulminating type of infection in the susceptible host sharply limits the possibilities of specific or other forms of therapy.

CONCLUSIONS

1. St. Louis encephalitis virus injected intracerebrally into susceptible mice multiplies there to reach a titre of 10^9 intracerebral lethal

doses. It is found also in the blood in small amounts immediately following injection and preceding death.

2. Injected intraperitoneally or subcutaneously the virus circulates in the blood for several hours and survives in the spleen for days. It does not multiply in the brain and cause encephalitis, however, unless overwhelming doses are injected or the brain is traumatized.

3. Virus dropped into the nares is demonstrable in the olfactory bulbs at 24 hours, in the piriform lobes at 24 to 48 hours, in the remainder of the brain at 3 days, and in the spinal cord at 4 days. In the brain it reaches a titre of 10^9 in 6 days. Virus is not readily demonstrable in the blood but is present in the spleen after 48 hours.

4. Virus survives and is capable of multiplying in the spleen.

5. Lesions following nasal instillation of virus appear first in the olfactory bulbs on the 3rd day, in the piriform lobes on the 4th, and in Ammon's horn on the 5th day. The character of the lesions in order of their appearance is exudation of mononuclear cells about superficial blood vessels and in the pia, hyperplasia of the endothelium of the pia, and necrosis of nerve cells of the olfactory tract.

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EXPLANATION OF PLATES

Sections through brains of susceptible mice at various intervals after nasal instillation of St. Louis encephalitis virus. Eosin-methylene blue stain.

PLATE 32

FIG. 1. 3 days. Olfactory bulbs. Ventral, medial, posterior surface. One of a few areas showing exudate of round cells and polymorphonuclear neutrophiles about the blood vessels in and just beneath the pia. A few leucocytes are at some distance from the blood vessels. × 675.

FIG. 2. 4 days. Piriform area. Ventral, medial surface. The pia is thickened. Round cells and a few polymorphonuclear cells are collected near the blood vessels in and beneath the pia and are scattered about the nearby superficial brain tissue. Superficial capillaries are distended. The deeper portions of the brain, including the nerve cells, appear normal. × 275.

PLATE 33

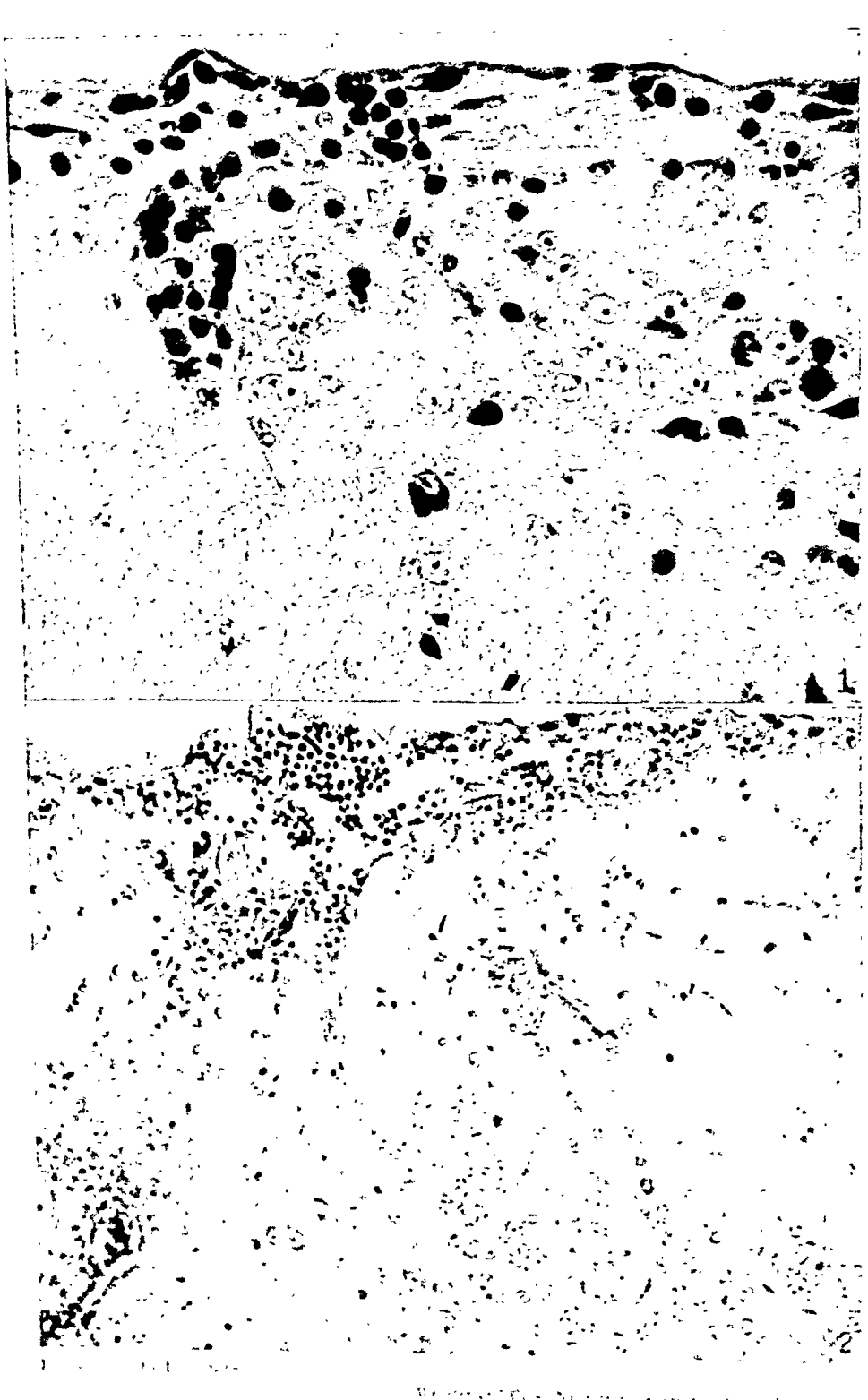
FIG. 3. 4 days. Same region. The changes described in Fig. 1 are shown in detail. × 675.

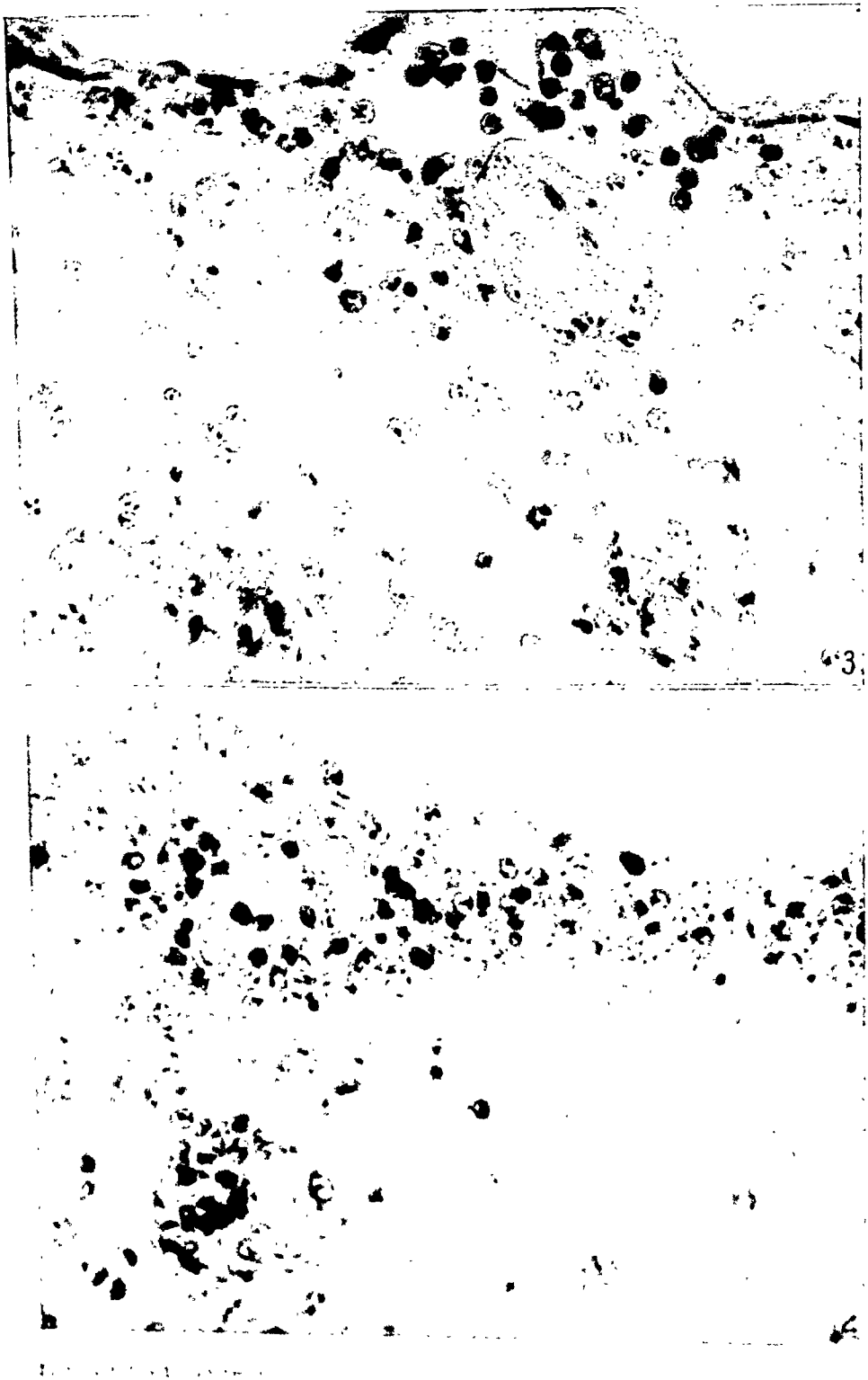
FIG. 4. 4 days. Same region. The pial endothelium is markedly hyperplastic and infiltrated with leucocytes from neighboring blood vessels. × 675.

PLATE 34

FIG. 5. 6 days. Ammon's horn. To the left of the figure, the nerve cells appear normal; to the right, they are in various stages of disintegration. The earliest alteration seems to be an increase in the affinity of the cytoplasm for eosin with a tendency to granulation. The cytoplasm of other cells still takes a deep eosin stain but is shrunken and surrounds a nucleus in various stages of pycnosis. Finally, the cell shrinks to a small, round, deep staining nucleus with little cytoplasm, and then seems to disappear, leaving the surrounding tissue vacuolated. An occasional nerve cell in the nearby matrix exhibits the same sort of degeneration.

FIG. 6. 7 days. Piriform area. Ventral, medial surface. The pial exudative and hyperplastic lesions are not conspicuous. The pyramidal nerve cells to the lower left of the figure are in various stages of necrosis. To the right, they are almost entirely absent, leaving vacuolated areas without inflammatory reaction. × 675.





STUDIES ON PSEUDORABIES (INFECTIOUS BULBAR PARALYSIS, MAD ITCH)

III. The Disease in the Rhesus Monkey, Macaca mulatta

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Plates 35 and 36

(Received for publication, October 30, 1935)

It has already been noted (Hurst, 1933) that the monkey (*Macaca mulatta*) is susceptible to intracerebral inoculation of either the Aujeszky or the Iowa strain of pseudorabies virus. The details of infection in this species appear of sufficient theoretical importance to warrant the brief description which is the object of the present communication.

EXPERIMENTAL

In England in 1930, using a strain of Aujeszky virus,[1] I was unable by intracerebral or intramuscular inoculation to secure infection of a *rhesus* monkey. Remlinger and Bailly (1933) also recorded failure with another species—*Innus ecaudatus*.

With the Iowa strain of pseudorabies virus, and with an Hungarian strain,[2] intracerebral injection at Princeton (1932) was successful in each of 5 cases. Inoculation by other routes met with less success. Intrasciatic inoculation accompanied by mild trauma (Hurst, 1930), and often by ligature of the nerve between the site of deposition of infective material and the point of entry of the needle (to prevent leakage), gave very inconstant results. Of 12 animals so inoculated with highly virulent rabbit virus, 2 only developed symptoms; the remainder were inoculated a second time in the nerve of the opposite

[1] Obtained through the kindness of Mr. I. A. Galloway.

[2] Obtained by Dr. R. E. Shope from Professor Aujeszky.

side, when one more animal became infected. The survivors, refractory to 2 intraneural inoculations and having throughout shown a normal temperature curve, possessed no antiviral substances in the serum. A 13th monkey injected on each of two occasions with monkey virus developed no symptoms, but was later found to resist intracerebral inoculation of rabbit virus and to possess neutralising bodies in the serum. Intramuscular and intravenous inoculation of massive doses (3 cc. and 5 cc. respectively of a 10 per cent suspension[3]) evoked neither symptoms nor immunity.

Returning to England (1934), I inoculated 2 more monkeys intracerebrally; both showed a temperature reaction without definite

TABLE I

Results of Inoculating Pseudorabies Virus into Monkeys Not Previously Tested for Immunity to B Virus

Route of inoculation	No. of monkeys inoculated	Developed nervous symptoms Killed for histology or when moribund	Temperature reactions No definite nervous symptoms Later immune serologically	No apparent illness Later immune serologically	No apparent illness Later not immune serologically
Intracerebral	7	5	2	—	—
Intrasciatic	13	2	—	1	9
Intrasciatic (reinoculation)	11	1	—		
Intramuscular	1	—	—	—	1
Intravenous	2	—	—	—	2

nervous symptoms, and later possessed neutralising bodies in the serum. These results are summarised in Table I.

At this point Sabin made the interesting observation of a partial antigenic relationship between the pseudorabies and B viruses (1934 *a*) and found that 1 of 13 apparently normal monkeys was immune to the latter (1934 *b*).

It therefore seemed desirable to ascertain whether immunity to B virus (Sabin and Wright, 1934) is an important factor in influencing the course of pseudorabies in the monkey.

Incidence of B Virus-Immune Monkeys.—As stated, Sabin found

[3] 1 cc. of 10 per cent suspension of the virus used represents 1 to 10 million intracerebral infecting doses for the rabbit.

TABLE II

Results of Inoculating Aujeszky Virus into Monkeys Tested for Immunity to B Virus

No.	0.5 cc. serum neutralised B virus	Route of inoculation	Result	Remarks
	M.L.D.			
6-16	>500	Intracerebral	T 4–12, R	Serum neutralized Aujeszky virus after, but not before experiment. Characteristic residual lesions in brain when killed
6-17	>500	"		—
6-19	>500	"	+5, D 8	As 6-16
6-20	>500	Intravenous	T 2–9, R	Later, no neutralising antibodies to Aujeszky virus
6-21	>500	"	Nil	As 6-20
6-22	>500	Intradermal	"	As 6-20. Later succumbed to intracerebral inoculation (+3, D 9)
6-18	<10	Intradermal	"	As 6-20
6-25	<10	"	Nil	As 6-20. Later succumbed to intracerebral inoculation (+5, D 9)
6-26	<10	"	"	As 6-20. Later succumbed to intracerebral inoculation (+4, K 5)
6-27	<10	Intracerebral	"	—
6-28	<10	"	+6, D 10	—
6-29	<10	"	+4, D 8	—
6-36	<10	Intracisternal	+4, D 9	As 6-16
6-37	<10	"	T 4–10, R	—
6-38	<10	Intrasciatic	+7, K 8	As 6-20
6-39	<10	"	Nil	—
6-40	<10	"	+20, K 21	As 6-16
6-41	<10	"	T 7–9, R	—
6-42	<10	"	+13, K	As 6-20
6-43	<10	"	Nil	As 6-16
6-44	<10	"	T 5–7, R	—
6-45	<10	"	T 6, K	As 6-20
6-49	<10	"	T 8, K	As 6-16
6-50	<10	"	T 8–10, K	Nervous system virulent
6-51	<10	"	T 8–10, R	As 6-44
6-52	<10	"	Nil	As 6-44
			T 8–9, K	As 6-16
				As 6-20
				As 6-44

T 4 = temperature on 4th day. No definite nervous signs.
+5 = typical nervous disease on 5th day (confirmed by histological examination).
K 6, D 6 = killed or died on 6th day.
R = recovered.

only 1 of 13 monkeys immune to B virus. It was, therefore, surprising to find that of the first 7 monkey sera tested, 6 contained abundant antibodies to this virus; the animals had all been obtained at one time from the dealer, and it is probable that they represented a single consignment from India. Of a second group of 5 monkeys and a third group of 14 obtained at later dates, not one furnished a neutralising serum.

Pseudorabies in Monkeys Tested for Immunity to B Virus.—The results of the renewed investigation are set forth in Table II. Although the observations are not sufficiently numerous to be of statistical value, they suggest that in the presence of immunity to B virus intracerebral inoculation of pseudorabies leads often to a non-fatal instead of a fatal illness. On the other hand, intracisternal inoculation of 1 cc. of 10 per cent pseudorabies virus is not invariably fatal even in monkeys possessed of no immunity to B virus. Recent results of intrasciatic inoculation (0.2 cc. of 10 per cent emulsion), always in animals not immune to B virus, were distinctly better than those on previous occasions. Intradermal inoculation (2 cc. of 10 per cent suspension distributed in ten areas) was without effect whether the monkeys were immune to B virus or not; as compared with areas inoculated in the same animals with normal rabbit brain no difference in macroscopic reaction was noted, and the temperature curve was only slightly disturbed immediately after the injection, presumably as a result of injection of much foreign protein. Intravenous inoculation (5 cc. of 10 per cent suspension prepared with fresh normal rabbit serum to eliminate the possibility of any toxic effect of the brain extract (Dold and Ogata, 1911, 1912)) was negative in 2 B virus-immune animals. Although infected animals were kept in cages closely adjacent to normal monkeys which picked their affected brothers, no contact infection ever resulted.

Symptomatology

Within 50 to 90 hours of intracerebral inoculation, 9 of 12 animals showed rises of temperature of from 1.5° to 3.5°F. lasting until nervous symptoms had reached an advanced stage. 3 cases which developed nervous symptoms and died (or were killed at an advanced stage) remained afebrile throughout. In all, the temperature fell to a subnormal level when death appeared imminent. On a number of occasions too numerous to be coincidental, the temperature became

subnormal about half way through the period of nervous symptoms, to rise again within 12 to 24 hours; this drop was accompanied by no amelioration of the clinical condition, and occurred both in cases showing nervous symptoms, and later dying, and in those suffering only from pyrexia and subsequently recovering. Nervous symptoms appeared at times ranging from 3 to 7 days after inoculation.

After successful intrasciatic inoculation the clinical course was very similar; the temperature usually rose on the 5th–8th day, with nervous signs supervening in most cases on the 11th–15th day; in one case, however, not until the 20th day.

Though not dramatic, the onset of nervous symptoms was sufficiently abrupt. From a state of normal vivacity and inclination for food the animal lapsed into one of apathy, but not somnolence, and could be roused only by sustained stimulation; often it was no longer aggressive on disturbance and paid no attention to food or to a cage mate. In many instances within the next 12 hours or so epileptiform fits ensued in increasingly rapid succession. A fixed stare and twitching of the eyelids, ears and mouth, often heralded by characteristic cries, preceded tonic and later clonic spasms first of the contralateral face and tongue, then of the contralateral arm, and finally of the whole musculature. Between the convulsions coarse lateral nystagmoid movements, sometimes associated with jerks of the head to the contralateral side, often persisted together with fibrillary twitches in the general musculature. "Tasting movements" of the jaws unassociated with the usual picking and eating of sebum were in some monkeys almost continuous. Almost as frequent were forced movements of greater or less extent. In their mildest form the head slowly wandered to one side, usually that of the inoculation, to be brought back by a sudden jerk; when more severe the head and body were forcibly twisted until the animal was compelled to complete the rotation around its vertical axis and thus return to its original position. When recumbent it rolled over and over on the floor of the cage. Some cases manifested constant, almost rhythmic, rolling movements of the head, which was successively flexed on the chest, rotated to one side, extended and returned to its former position; or sliding movements of the arm across the body, with flexion at the elbow and extension of wrist and fingers, recurred frequently. Mild, moderate or marked salivation was present. The pupils were small and reacted briskly to light.

In severe cases, with the passage of a further 24 or 48 hours the monkey was semicomatose; more or less generalised muscular twitching continued or status epilepticus supervened. The pupils were now dilated and insensitive to light. There was great general weakness but no localised paralyses, except once double ptosis.

The foregoing clinical picture was not invariable. Fits might mark the onset and the animal thereafter remain free, to die suddenly a few days later. Twitching of the face might be the only spasmodic element. Sometimes great general weakness without local paralyses or fits obtained from the onset. Animals presenting only fever later showed unmistakable residual lesions (see below) in the

characteristic situations; of course, it is realised that in these cases rare fits might have occurred during periods when the animals were not under observation. Such animals made an apparently complete recovery except in one case in which the monkey manifested a change in temperament, becoming extremely tame and docile though prior to the experiment it had been just the reverse. Once nervous symptoms were definite, recovery never occurred.

Distribution of Virus

Four monkeys, one dying of tuberculosis on the 4th day and the others sacrificed with advanced nervous symptoms on the 6th, 6th and 9th days respectively after intracerebral inoculation, showed no virus in the defibrinated blood when 10 cc. amounts were injected subcutaneously into rabbits. The cerebrospinal fluid in 2.5–5.0 cc. amounts was likewise uninfective. The lungs, livers, spleens, kidneys, adrenals, salivary glands and cervical lymph glands in doses of 1 cc. of 10 per cent suspensions all proved innocuous. Both cerebral hemispheres of each case were virulent, as were, with long incubation periods denoting the presence of a minimal amount of virus, the lumbar cords. The sciatic nerves were not virulent.

Four other intracerebrally infected monkeys were bled daily from the beginning of the experiment, and defibrinated blood in amounts of 2–3 cc. inoculated subcutaneously into rabbits with uniformly negative results.

Table III illustrates the spread of virus in the nervous systems of monkeys infected by the intrasciatic route. The cerebrospinal fluid appears to take no part in its dissemination. The lumbar cord is the first part of the nervous system to become infective, and the cervical cord and medulla are virulent before the cerebral cortex. At a given period lesions in the lumbar cord are more advanced than in the cortex, yet at the same time are always minimal in intensity (see below). When the characteristic cortical disease is present the lumbar cord may be no longer infective, especially in cases of slow evolution, though sections show slight lesions indicative of the previous activity of the virus. These facts are interpreted as indicating that the cells of the lumbar cord are relatively insusceptible to the virus, which does not readily gain a foothold here and tends soon to die out. Parts of the cerebral cortex representing sites of election of severe lesions (see below) become infective before areas seldom or never affected

(*e.g.*, the lateral occipital cortex), and judging by the duration of the incubation period in passage animals the latter when virulent contain only small amounts of virus. In a single case the motor cortex corresponding to the inoculated nerve was virulent before that of the opposite side (*cf.* observations in poliomyelitis; Hurst, 1930), but in view of the complexity of the nerve paths from the lumbar cord to

TABLE III

Distribution of Pseudorabies Virus in the Nervous System of Monkeys Following Left Intrasciatic Inoculation

No.	Days after inoculation	Symptoms	Presence of virus in											
			Cerebrospinal fluid 2.5–5.0 cc.	Lumbar cord	Cervical cord	Medulla	Right anterior frontal cortex	Right motor cortex	Right temporal cortex	Right occipital cortex	Left anterior frontal cortex	Left motor cortex	Left temporal cortex	Left occipital cortex
6-44	6	Temperature 1°F. above previous level	0	+5	0	0	0	0	0	0	0	0	0	0
6-45	8	Temperature rising sharply from previous level	0	+4	+4	+3	0	0	0	0	0	0	0	0
6-49	10	Pyrexia for 3 days; no nervous symptoms	0	+8	+4	+3	+4	0	0	0	0	0	0	0
6-52	9	Pyrexia for 2 days; no nervous symptoms	0	+4	+4	+3	+8	0	+3	0	+4	0	0	0
P 12	15	Pyrexia for 2 days; early nervous symptoms	–	0	–	+11	0	+3	+3	0	+5	0	+4	0
P 7	12	Nervous symptoms for 24 hrs.	0	+3	+5	+5	+3	+4	+3	+5	+3	+5	+3	+8
6-39	21	" "	0	0	–	+3	–	–	+2	–	–	–	+3	–

+ = development of pseudorabies in passage rabbit with incubation period in days.

0 = no take.

– = not tested.

The areas of cortex chosen for passage were as follows: (*a*) anterior frontal—frontal pole including part of basal surface; (*b*) motor cortex—upper part including leg area and part of medial surface of hemisphere to cingular sulcus; (*c*) temporal pole—including pyriform area and anterior extremity of cornu Ammonis; (*d*) occipital cortex—smooth lateral surface anterior to occipital pole.

other parts of the cortex examined it is not perhaps to be expected that a clear cut unilateral distribution would obtain at an early stage of cortical involvement.

Histological Examination

1. Lesions Following Intracerebral Inoculation.—The essentials of the histological picture following injection by this route have already been described (Hurst, 1933), and certain points regarding the distribution of the lesions alone require emphasis.

Greater experience permits more definite statement of the parts of the nervous system chosen for the major attack. These are in order of frequency and of severity of affection (*a*) the cornu Ammonis or the pyriform area, or often both together, (*b*) parts of the cerebral cortex bordering on the Sylvian fissure, particularly the lower lip of the fissure and the anterior part of the island of Reil, (*c*) much less frequently and less severely other parts of the cerebral cortex, especially the basal surface of the frontal lobe, parts of the temporal cortex not mentioned above, and the lips of the cingular sulcus. The basal ganglia and brain stem suffer to a variable but never considerable degree; vascular and tissue infiltration are here more conspicuous than is extensive nerve cell destruction. The cerebellum is usually intact. In the cerebrum the attack of the virus appears to be on the selected areas as areas, and not on a particular cell type—thus in the cornu Ammonis the greater part of the pyramidal cell band may be necrotic and the end-plate and fascia dentata spared, or the fascia dentata may suffer more than the pyramidal cells, or the cells of the end-plate may be chiefly affected. At a significantly early stage there may be no meningeal or vascular damage; the picture is then unequivocally that of a primary attack on the nerve and glial cells. After intracisternal inoculation the same distribution obtains.

Residual Lesions.—In the first day or two of the nervous disease, the absence of tissue and vascular reaction makes the detection of lesions in all but the most severely affected areas a matter of rather painstaking study with a high power of the microscope. Later, the secondary inflammatory reaction increasing rapidly in intensity causes the sites of election of nervous destruction to leap to the eye, as the series of photographs here reproduced demonstrates. In the most severely affected areas of non-fatal cases the tissues may ultimately be

reduced to porencephalic cavities, or where the injury has been rather less severe a dense glial-mesodermal scar may develop. Perivascular and meningeal infiltration persists for months after the initial injury. In these later stages definite macroscopic changes, previously absent, are also noted; the earliest is considerable congestion of the stricken areas first visible about 4 days after the onset of nervous symptoms. The following cases are typical.

M 6-16. 3 weeks after intracerebral inoculation in a monkey (serologically) immune to B virus. Pyrexia from the 4th–12th day had been accompanied by no definite nervous symptoms other than great general weakness. At death the serum neutralised pseudorabies virus.

At autopsy both temporal poles were distinctly soft to palpation. They looked pale, yellowish and opaque as contrasted with other areas of the brain, and the finest vascular markings were absent. On approaching the damaged area the larger vessels seemed to diminish in calibre, probably because a small amount of greyish translucent exudate obscured them in the sulci. A few brownish flecks probably represented old pial haemorrhages. On section after fixation in formol-saline, the grey matter of the cortex appeared to end on reaching the curve of the temporal pole, where it gave place to a wider, yellowish, opaque zone in which demarcation between the grey and the white matter proved impossible. Photographs show far better than does a lengthy description the distribution of residual lesions in the brain (Figs. 1–4). The cortex of the temporal pole and the cornu Ammonis were in large part completely destroyed; the remaining structures consisted almost wholly of hyperplastic and infiltrated pre-existing and new-formed vessels; granular corpuscles and infiltrating lymphocytes and plasma cells (scanty); and many hypertrophied glial cells where the intensity of the destruction was slightly less. There was a similar scar at the anterior end of the island of Reil, and smaller areas in the lips of the Sylvian fissure and superior temporal sulcus. Elsewhere in the cortex and basal ganglia lesions were minimal; in the walls of the third ventricle and in the brain stem they were only slightly more severe.

M 6-36. 3 weeks after intracisternal injection. Pyrexia on the 4th–10th days after injection and transient nervous symptoms on the 8th day were the chief clinical features. At death the serum neutralised pseudorabies virus.

Residual lesions did not compare in severity with those in the preceding case; they were most intense in the pyriform area of the temporal lobe, especially on one side, slight around the Sylvian fissure, and of intermediate severity in the walls of the third ventricle. The brain stem was not examined.

M 6-06. 21 months after intracerebral inoculation which was succeeded on the 3rd–10th days by pyrexia but no nervous symptoms other than general weakness. Later the temperament of this animal was quite altered; the serum contained neutralising antibodies.

On palpation both temporal lobes gave the impression of softening; microscopi-

cally severe residual lesions were seen. Anteriorly, the meninges over the temporal poles were thickened and infiltrated with mononuclear cells. In the subjacent cortex marked perivascular sheathing, diminution or absence of nerve cells, and increase of glia and microglia (chiefly granular corpuscles) marked the less affected parts, while the more affected areas were reduced to small porencephalic cavities separated by a scanty framework of glial-mesodermal scar tissue (Fig. 5). A similar much smaller area lay in the anterior part of the island of Reil, and still less severe lesions in the cortex forming the lower lip of the Sylvian fissure. There was some residual meningeal infiltration in one part of the cingular sulcus.

Farther back all structures of the cornu Ammonis were wanting except in its most posterior part behind the level of the thalamus; the descending horn of the lateral ventricle was greatly dilated and separated from the meninges by a narrow strip of sclerotic tissue only slightly exceeding 1 mm. in width (Fig. 6). No other lesions were noted.

M 6-05. 4 months after intracerebral injection. No definite pyrexia or nervous symptoms followed the inoculation, but the serum later contained neutralising antibodies. Residual lesions were less extensive than in the preceding animal.

Macroscopically there was some shrinkage in the cornu Ammonis and adjacent regions. The chief destruction was of the pyramidal cell band of the cornu Ammonis, which over more than half of its extent including the whole expansion within the curvature of the fascia dentata was completely replaced by a glial-mesodermal scar. The fascia dentata was intact. Less severe changes (diminution in the number of nerve cells, increase of glia and residual perivascular cuffing) were present in parts of the temporal cortex anterior to the cornu Ammonis. Meningeal infiltration persisted in the Sylvian fissure and superior temporal sulcus.

2. Lesions Following Intrasciatic Inoculation.—The distribution of lesions following intrasciatic inoculation is of importance; the 8 cases examined histologically were sufficiently similar to obviate the necessity of separate description.

At the site of inoculation in the sciatic nerve wholly non-specific degenerative and reparative changes proceeded. In no case at the time of autopsy were nuclear inclusions present.

According to the period at which the monkey was killed, the spinal ganglia corresponding to the inoculated nerve showed either occasional necrotic nerve cells, with chromatolysis and mild degenerative changes in a proportion of the remainder, or else moderate mononuclear interstitial infiltration (with perhaps a few polymorphonuclear leucocytes or plasma cells) and foci of proliferated capsule cells replacing vanished neurons. The ganglia of the opposite side presented less severe lesions or none at all; those examined from higher levels were normal.

In the anterior horn cells of the lumbosacral cord of some cases, axonal reaction attributable to ligature of the inoculated sciatic nerve had to be distinguished from lesions resulting from virus activity. The latter consisted in necrosis of occasional nerve cells and their replacement by phagocytic cells; a few focal collections of microglial cells, particularly in the posterior and anterior horns of the inoculated side; moderate or mild mononuclear cuffing of the larger vessels; and in some cases, but not in all, slight mononuclear infiltration in the meninges.

In the dorsal and cervical cord at different levels and in different cases lesions might resemble the above in severity or be almost or wholly non-existent.

Above the foramen magnum lesions were wholly comparable with those following intracerebral inoculation. The outstanding nervous destruction was limited to the regions already indicated, and in many cases the degenerative process in the nerve cells again progressed to necrosis in the complete absence of meningeal infiltration, or of any abnormality, inflammatory or otherwise, of the blood vessels.

Finally, histological examination supported the belief that the spread of virus is from below upwards. For in cases in which early lesions were present in the lumbar cord and ganglia, the temporal cortex, etc., were still free from change. While when the temporal cortex was strewn with recently necrotic cells or cells bearing nuclear inclusions, but before tissue, vascular and meningeal reaction had appeared here, the lumbar cord and ganglia were the seat of definite reactive inflammation, many or all the dead nerve cells had been removed, and nuclear inclusions had disappeared.

To summarise, the histological findings agree with the experimental evidence in suggesting an upward spread of the virus, after intrasciatic inoculation, presumably by the nervous path. Although virus enters by the lumbar cord and might be expected to produce its maximal effects here, the striking localisation of the most severe lesions to the temporal lobe, etc., is once more observed, and must be regarded as characteristic of the action of pseudorabies virus in the monkey. The virus evidently possesses relatively little affinity for the neurons of the spinal ganglia, cord, brain stem, cerebellum and basal ganglia. On the contrary certain areas of the cerebral cortex are peculiarly vulnerable to its action, and the result is a selective distribution of lesions as insusceptible of facile explanation as that in poliomyelitis or louping-ill.

3. Lesions Following Intradermal Inoculation.—It has been recorded (Hurst, 1933) that in pseudorabies in the monkey nervous system nuclear inclusions are seen only in nerve and (less often) glial cells, and never in mesodermal cells of the vessels and meninges. In the rabbit, on the other hand, nuclear inclusions occur in cells derived

from all three embryonic layers (Hurst, 1934). If the virus be introduced into the skin of the rabbit or pig a focus of necrosis accompanied by much local multiplication of virus results.

In the present work monkeys were inoculated intradermally in many areas with pseudorabies virus, and with normal rabbit brain for purpose of control; daily from the 1st–6th days skin was excised for histological examination. In no instance were the appearances with pseudorabies virus different from those with normal rabbit brain. Inclusions were never seen. In the disease provoked by intracerebral or intrasciatic inoculation virus and lesions are absent from the general viscera. There is, therefore, no cytological or other evidence that in the monkey the pseudorabies virus can attack any but neuro-ectodermal cells.

DISCUSSION

Two points of interest emerge from this study. The first concerns the different behaviour of the virus of pseudorabies in various animal species. While in the rabbit it is strongly neurotropic, its activities do not end here; in this animal it is capable of multiplying and producing specific lesions in a great variety of cells derived from all three embryonic layers. In other words, it can be described as pantropic (Hurst, 1934). In the monkey, on the other hand, it appears to have no affinity for any cells other than nerve and glial, and in its behaviour under experimental conditions resembles purely neurotropic viruses like rabies and poliomyelitis. In future, in qualifying a virus by some term indicative of its cellular affinities, it will be necessary to specify clearly the animal species on which observations have been carried out.

Most of the common domestic and laboratory mammals exposed to pseudorabies virus readily contract the disease and die; two stand out as possessed of greater resistance, *viz.* the pig and the monkey. In stating that the virus is less virulent towards these species, or alternatively that they are less susceptible to its action, it is well to realise that in the two instances the mechanism of resistance is wholly different.

Inoculated subcutaneously the pig develops a definite local lesion (Hurst, 1933), associated presumably as in the rabbit[4] with great

[4] Unpublished observations.

multiplication of virus; only small amounts are necessary to infect by the skin (Köves and Hirt, 1934). During the course of the infection virus is present in the blood and blood-rich organs (Köves and Hirt); lesions may be present in the lymph glands, spleen and heart (Hurst). Virus invades the nervous system where, however, there is no great outfall of nerve cells (Hurst), with the consequence that definite nervous symptoms are rare (Shope, 1931) occurring probably in less than 10 per cent of the cases (Köves and Hirt); in the absence of the characteristic biting and scratching, the disease is often wrongly diagnosed, if indeed without regular temperature readings it is diagnosed at all. Almost always the pig recovers and is subsequently immune; Köves and Hirt place the mortality rate at less than 5 per cent. But the nasal secretions become infective (Shope, 1934, 1935) as does the urine (Köves and Hirt), and the pig transmits the disease to its fellows and to other animals. In short, in the pig the virus exhibits multiple tissue affinities; the animal very easily contracts the disease, but survives because its nervous system is relatively resistant and is not overwhelmed unless a considerable dose of virus be given directly into the brain.

By way of contrast, large doses of highly virulent rabbit brain introduced intradermally into the monkey evoke no more reaction than does normal brain tissue; intramuscular and intravenous inoculation are probably equally fruitless. No infection follows, so that subsequently the animal is not immune. But if virus be brought into sufficiently intimate contact with nervous tissue, as by intrasciatic inoculation, it may invade the nervous system to set up a disease accompanied by clinical symptoms and fatal in many cases. During this disease virus does not circulate in the blood and the animal does not infect a cage mate; no virus or lesions are present in the general viscera. In brief, in the monkey the nervous system is probably less resistant to infection than in the pig, but since the virus behaves in this species as a strict neurotrope it does not readily reach the susceptible tissue, destruction of which is incompatible with life. Another factor in the resistance of the monkey is, no doubt, the relative insusceptibility of the lower regions of the nervous axis; were the highly susceptible neurons in this instance those of the anterior horns of the cord as they are in poliomyelitis, instead of those of the cerebral cortex not easily reached from the periphery, intramuscular and intradermal

deposition of virus, even though not followed by local multiplication, might be more effective in producing nervous infection.

The last observation is the second point worthy of comment. Although in pseudorabies in the monkey the most severely affected areas of the cortex are roughly those underlying the most deeply coloured parts of the meninges after intracisternal inoculation of dyestuffs (Hurst, 1932), we cannot explain the present findings solely, if at all, on the basis of dissemination of the virus by the cerebrospinal fluid. Not only was the virus never found in the fluid, even at an early stage after intrasciatic inoculation, but the correlation between the distribution of lesions in pseudorabies and that of colouring matter introduced into the fluid is far from complete if the nervous system as a whole is considered. We know already of two virus diseases of the central nervous system in which, for reasons at present obscure, a particular type of nerve cell is involved far more severely than others; these are poliomyelitis in man and the monkey, and louping-ill in the monkey (Hurst, 1931; Findlay, 1932), with respectively motor neuron and Purkinje cell involvement far surpassing that of other cell types. In the present instance certain cortical areas seem to be selectively attacked, though within these areas more than a single cell type is included in the destruction. Whatever the underlying cause, this distribution of lesions appears at present to be highly characteristic of pseudorabies in the monkey.

CONCLUSIONS

In the monkey (*M. mulatta*) the virus of pseudorabies, pantropic in the rabbit, behaves as a strict neurotrope. Infection, usually fatal, readily follows intracerebral and intracisternal inoculation of rabbit virus, and often intrasciatic inoculation; the symptomatology of the ensuing disease is described. In a limited number of experiments no infection resulted from intradermal, intramuscular or intravenous inoculation. Nerve and glial cells are primarily attacked by the virus, but no cytological or other evidence of susceptibility of non-nervous tissue or of growth of virus outside the nervous system was obtained. Certain cortical areas, of which the principal are the pyriform area, cornu Ammonis, island of Reil, lower lip of the Sylvian fissure and basal surface of the frontal lobe, are affected far more severely than are other parts of the nervous axis; the reasons for this elective dis-

tribution of the most severe lesions, seen alike after intracerebral and intrasciatic inoculation and analogous perhaps to that in poliomyelitis and louping-ill, are not obvious. Other areas of the nervous system are relatively insusceptible to the action of the virus. Cases showing clinically only a febrile reaction without definite nervous symptoms may later exhibit marked residual lesions at the sites of election. The blood and cerebrospinal fluid play no apparent rôle in disseminating the virus, which, after intrasciatic inoculation, spreads upwards by the nervous path.

Some suggestion was received from the experiments that in monkeys possessed of immunity to B virus (Sabin and Wright, 1934) pseudorabic infection is less likely to prove fatal than in animals not so immune, but the observations made were insufficiently numerous to be of statistical value.

The sera of 6 out of 26 monkeys were found to contain antibodies neutralising B virus; these 6 monkeys were all included in one batch of 7 received at one time from the dealer.

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EXPLANATION OF PLATES 35 AND 36

FIGS. 1 to 4. Residual lesions in the brain of M 6-16. The intensely stained areas represent tissue and perivascular infiltration at the sites of maximal damage. The distribution is outlined in the text. Haematoxylin and Van Gieson. × about 3½.

FIGS. 5 and 6. Residual lesions in the brain of M 6-06. The former shows the porencephalic softening at the temporal pole, the latter the shrinkage of cerebral tissue with complete absence of the cornu Ammonis and compensatory dilatation of the lateral ventricle. Haematoxylin and Van Gieson. × about 3½.

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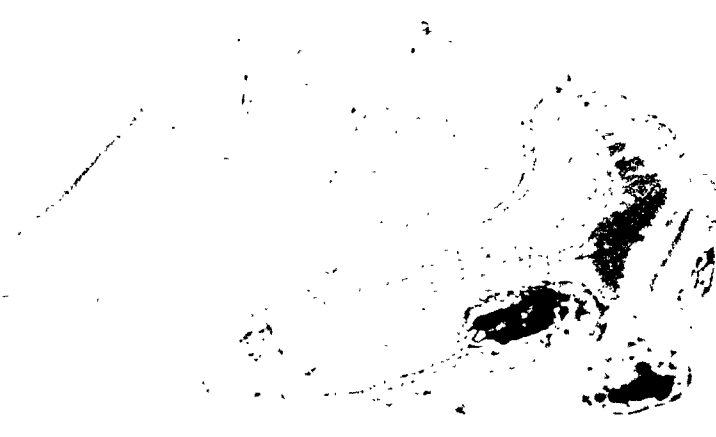

4

THE EFFECTS OF ADRENALECTOMY AND HYPOPHYSECTOMY UPON EXPERIMENTAL DIABETES IN THE CAT

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The possible participation of the adrenal glands in the series of events following pancreatectomy in animals has been the subject of numerous investigations. These fall into two main groups: (*a*) those in which the adrenal medulla was removed or its secretion paralyzed by section of the nerve supply to the gland before or after pancreatectomy, (*b*) those in which both adrenal glands were removed along with the pancreas. The order in which these different endocrine glands were excised has varied all the way from attempts to remove both adrenals and pancreas at one operation by Mayer (1) to the serial operations used by Hédon (2).

As the result of the first group of experiments there has accumulated the most convincing evidence that the withdrawal of epinephrine from the body either by removal of the adrenal medulla or section of its nerves does not in any way modify the effect of a subsequent pancreatectomy (3–8).

It is obvious that great difficulties confront the attempt to remove both adrenal glands and the pancreas, and these difficulties are reflected in the results of many published experiments. Until recently the short survival and moribund condition of such preparations has rendered almost useless any observations that have been made. Stewart and Rogoff (4) have severely criticized all experiments made up to 1922 which purported to show some interrelationship between these endocrines, but it must be stated that their conclusion that the adrenals do not influence pancreatic diabetes is not supported by their own experiments, since their totally adrenalectomized and depancreatized dogs lived only a few hours.

Turcatti (5) has prepared 11 totally adrenalectomized and depancreatized dogs. The operations were carried out in various orders. The shortest survival was 5½ hours and the longest 40 hours. Nevertheless, this author concludes that simultaneous removal of all adrenal and pancreatic tissue prevents the usual hyperglycemia. The paper of Viale (10) appears to be a recitation of the experiments of Turcatti (5). In another paper by Lewis and Turcatti (9) one adrenal and the pancreas were removed and the dogs maintained with insulin. Upon

removal of the last adrenal all insulin was withdrawn. Of 11 dogs prepared in this manner the shortest survival was 11 hours and the longest 72 hours. Since but little change occurred in the blood sugar and as glycosuria persisted, the authors concluded that total adrenalectomy under these conditions was of no value in the alleviation of pancreatic diabetes.

The introduction of adrenal cortical extracts capable of maintaining life in totally adrenalectomized animals has been utilized by two groups of workers. Leloir (8) has prepared 4 dogs in the manner used by Lewis and Turcatti, but after removal of the last adrenal and the withdrawal of insulin the dogs were injected with a cortical extract prepared by the method of Swingle and Pfiffner. The longest survival was 3 days, too short a time to throw any light upon the influence of adrenalectomy in experimental diabetes.

While our work was in progress Hartmann and Brownell (11) published a preliminary report upon 7 cats that were totally adrenalectomized and depancreatized in stages. 2 died within 24 hours and the others were treated with cortin. Of these one died in 4 days without much reduction in the diabetes, another in 8 days, probably as the result of an insulin injection. Of the remaining 3, one died in 6 days during which period it exhibited lowered blood sugar and slight glycosuria. Another lived 13 days and exhibited some reduction in the diabetes. The last animal survived for 38 days and the authors state that for 3 weeks no reduction in the diabetes was observed, but after this a marked amelioration was found. It is obvious that the survival of a totally depancreatized cat for this period is quite exceptional. The authors conclude that cortin is necessary for the development of the full diabetic condition.

Throughout this work we have endeavored to make a quantitative comparison of several aspects of the diabetic syndrome following pancreatectomy in normal, hypophysectomized and adrenalectomized animals. We have been fortunate in being able to prepare a sufficient number of doubly operated animals whose period of survival and general condition warranted studies upon their metabolism and hence have not had to base our conclusions, as so many workers have done, upon animals which survived only for a few hours after operation.

Methods

Cats have been used in the experiments described. The reasons for using this species are: (*a*) the cat was the animal most readily available for pancreatectomy; (*b*) surgery is easy for mechanical reasons; and (*c*) because cats seem relatively resistant to shock and infection.

In all types of operation nembutal (pentobarbital sodium) has been the anesthetic. The intraperitoneal dose for the normal cat is 50 mg. per kilo. In operating on previously hypophysectomized or adrenalectomized animals, 30 mg. per kilo is sufficient. These animals must be anesthetized with great care.

All operations have been performed under strict aseptic technique except in the case of transbuccal hypophysectomies. Pancreatectomy has been done by dissection with fine forceps. In one series of animals two stage pancreatectomy was done by the removal of all but the splenic tip at a first operation, the remnant being removed subsequently.

Adrenalectomy, usually done through the lumbar approach, is very easy in the cat and needs no comment.

In addition to removal of the adrenals two other types of adrenal operation have been performed: (*a*) Denervation of the adrenal. After recovery from right adrenalectomy the left adrenal was exposed and the splanchnic nerve cut. The adrenal ganglion was also doubly ligated and cut. (*b*) Demedullation of the adrenals. After recovery from right adrenalectomy the left adrenal is denervated as above and the upper pole of the gland cut off. The adrenal medulla is then destroyed by a dental drill. The operation has been described by Houssay and Lewis (3) and others.

Hypophysectomy in the cat has been described by McLean (12), Allan and Wiles (13) and McPhail (14). We have used the buccal route, essentially as described by the above authors. The danger of infection is negligible. We have not used bone wax in the sphenoid opening and only the palate requires catgut sutures.

McPhail does not refer to unsuccessful hypophysectomy and it is worth mentioning that of the 11 fatalities occurring in 33 cats following buccal hypophysectomy, 7 were due to hypoglycemia on the 5th to 17th day. Meningitis occurred only once, accidental hyperpyrexia killed 2, and one had a large clot in the fossa. The surviving 22 cats were depancreatized. Of these 8 died from various causes soon after the operation. In other words, 14 animals, or 42 per cent, survived these two operations long enough to be considered successful preparations.

Care of Animals.—When first obtained, the animals have been kept and well fed in an isolation room for 1 to 2 weeks to reduce the incidence of respiratory infections. After first stage operations (first adrenalectomy or hypophysectomy) they have been kept in single cages which were carefully cleaned, and fed unmeasured amounts of raw beef and fish. Animals after pancreatectomy, with or without other operations, have been kept in metabolism cages and fed measured diets. The diets have been 40 gm. raw pancreas and 100 gm. of canned salmon, liver or beef daily. These diets have been varied at times and any food not eaten has been weighed and the actual intake determined. Water was supplied *ad lib.* These diets will maintain normal cats in good health. The animals were weighed at suitable intervals. The urine has been collected for each 24 hour period, preserved with toluol and kept in an ice box until the chemical studies were completed.

Chemical Methods.—Quantitative urine sugars have been done by Benedict's method and the results checked at intervals by the Shaffer-Hartmann method. Urine nitrogen has been determined by the micro Kjeldahl method and urine ketones as described by Van Slyke. Blood was obtained from the ear veins and

analyzed for glucose by the Benedict micro method. Blood for urea analyses may be obtained in the same manner.

Autopsy Findings.—All animals have been carefully autopsied and especial search made for pancreatic tissue. In the animals considered in this paper none was found.

In the adrenalectomized-depancreatized cats the presence of accessory cortical tissue was looked for. In only one animal out of 17 reported in this paper was any found. This cat (No. 2-20) will be referred to later.

The contents of the pituitary fossa of the 6 hypophysectomized-depancreatized cats described here were examined microscopically for hypophyseal tissue. In 3 animals fragments of the posterior lobe were found. In no case was any anterior lobe tissue present.

The Maintenance of Adrenalectomized Cats with Cortical Extracts

We have found that large amounts of cortical extract in terms of dog units a day are necessary to maintain adrenalectomized cats in good health.

In the present work we have used extracts from six sources. These were eschatin—Parke, Davis (10 dog units per cc.); a preparation of Dr. G. A. Harrop (50 dog units per cc.); an extract prepared by Dr. J. J. Pfiffner (60 dog units per cc.); a cortical extract supplied by Dr. G. F. Cartland of the Upjohn Company (130 dog units per cc.); an extract supplied by Dr. S. W. Britton (not assayed in dog units); and our own preparation by the Swingle and Pfiffner method (not assayed).[1]

It has been found that between 30 and 50 dog units per kilo of body weight per day are necessary under the conditions present in our laboratory to maintain adrenalectomized cats in good health. All the animals were adrenalectomized in two stages. They did not receive any additional sodium salts with the diet. In some of the animals receiving a large number of dog units daily the adequacy of the replacement therapy is well shown by the fact that they lived in excellent health and were later successfully depancreatized.

Classification of the Animals Studied.—The animals reported in this paper fall into five main groups. None of these received any insulin except where specially noted, but all the adrenalectomized animals received cortical extract daily.

Group 1.—At a preliminary operation all but one-sixth of the pancreas was removed together with one adrenal gland. The remaining splenic portion of the

[1] We are indebted to Drs. O. Kamm, G. A. Harrop, J. J. Pfiffner, S. W. Britton and G. F. Cartland for generous supplies of these extracts.

pancreas was either transplanted under the skin of the abdomen, or in some cases was left *in situ*. At a subsequent operation 10 to 34 days later the remainder of the pancreas and the second adrenal were removed. As controls upon this group other animals were subjected to similar procedures, except that at the completion of the pancreatectomy the remaining adrenal gland was left intact.

Group 2.—Total pancreatectomy was performed and the animals maintained by means of insulin and weighed diets. The adrenal glands were then removed in stages, insulin therapy being continued after the first adrenalectomy. After the second adrenalectomy all insulin was stopped. This method of preparing adrenalectomized-depancreatized animals has been the least successful of those that we have attempted, since all but one of the animals died within 3 or 4 days.

Group 3.—One adrenal was first removed. A week or 10 days later the second adrenal and the entire pancreas were removed. This operation is surprisingly well borne by the cat. 10 cats were operated upon in this manner.

As controls for this group we have used 10 cats, totally depancreatized in one stage, in which the adrenal glands were left intact. Furthermore, since there is a difference of opinion as to the effects of adrenal denervation and removal of the adrenal medulla upon pancreatic diabetes, we have depancreatized two further groups of 5 cats each. In the first group one adrenal was removed and the other denervated, in the second group one adrenal was removed and the medulla of the other gland drilled out, and the splanchnic nerve cut.

Group 4.—5 adrenalectomized cats maintained for various periods on cortical extracts prepared by different workers were depancreatized. One of these animals was exhibiting signs of adrenal insufficiency at the time of the pancreatectomy and died within 24 hours. The others were in excellent health and had held or gained weight since the total adrenalectomy.

Group 5.—Houssay and Biasotti (15, 16) have shown that a previous hypophysectomy greatly ameliorates the diabetes following pancreatectomy in the toad and dog. We have extended the number of species in which this effect is observed by depancreatizing 13 hypophysectomized cats. 4 of these died in diabetic coma following the injection of anterior pituitary extract (Squibb) at various times after pancreatectomy. One animal was also adrenalectomized, one killed for liver glycogen and in another cat the hypophysis was removed after the pancreas. The remaining 6 have been used for comparison with the totally adrenalectomized-depancreatized cats.

In assessing the effects of a given procedure upon pancreatic diabetes it is obvious that more than one criterion is desirable. Thus, prolongation of life is by itself not entirely acceptable especially if the period is only moderately extended. The individual variation may be so wide as to cast doubt upon the allegedly beneficial procedure. Nevertheless, if along with a moderate extension of the life span we find that the other stigmata of diabetes are also moderated, then we

are justified in assuming that the procedure employed has produced an alteration in the characteristic metabolic picture that follows total pancreatectomy.

Consequences of Pancreatectomy

The response of these various groups of animals to pancreatectomy has been examined in the following particulars:

Survival.—In studies upon experimental diabetes mellitus, the depancreatized dog has been the animal usually employed. By comparison, but few observations have been made upon the response of the cat to sudden deprivation of insulin. It has been recognized that in this animal the period of survival is much less than in the dog. In a study of 12 cats Azodi (17) found the average survival to be 4 days with a range from 2 to 5 days. Loewi (18) and Epstein and Baehr (19) observed a similar span of life after total pancreatectomy. Ring and Hampel (20) have remarked upon the rapidity and severity of diabetic acidosis and ketosis in this species following pancreatectomy.

Table I shows that the average survival of cats deprived of insulin is about 4 days. The manner in which this insulin deprivation is brought about does not appear to affect the survival period. Thus, cats acutely depancreatized or those in which a pancreatic remnant is removed, or those from which insulin therapy is abruptly withdrawn, all show an average survival of about the period stated. The extreme range of survival observed in any animals of this type is 2 to 8 days. In fact, 16 of the 19 animals in Table I, groups I A, II A and III A, were dead in 5 days or less.

Ring (21) has suggested that the amelioration of experimental diabetes produced by hypophysectomy or adrenalectomy might be attributed to the loss of weight suffered by the animals as a result of operations previous to the pancreatectomy. A study of Table I would indicate that this is not a factor in the cat. It is true that animals carrying a pancreatic transplant or those maintained upon insulin (Table I, groups I A and II A) lost about 20 per cent of their initial body weight before pancreatectomy. Nevertheless, when deprived of all insulin they did not survive longer than the animals in group III A in which total pancreatectomy was performed in one stage, when they were of normal weight.

Some workers have assigned to epinephrine an important rôle in the sequence of events that follows pancreatectomy. Indeed, Barnes and his coworkers (22) have recently published experiments which would indicate that removal of epinephrine from the organism by section of the splanchnic nerves or demedullation of the adrenals greatly ameliorates the diabetes following total pancreatectomy in the dog. Such animals are stated to survive longer and to exhibit a degree of glycosuria comparable to that found in the hypophysectomized-depancreatized dog. Results of this kind are in complete disagreement with

TABLE I

		No. of animals	Average weight loss		Survival	
			Before pancreatectomy	After pancreatectomy	Average	Range
			per cent	*per cent*	*days*	*days*
Group I	A. Pancreatic graft removed. Adrenals intact	3	18.0	9.0	3.3	2–5
	B. Pancreatic graft and both adrenals removed	6	22.0	5.0	8.7	6–12
Group II	A. Depancreatized. Insulin withdrawn	6	20.0	15.0	4.3	2–8
	B. Depancreatized, second adrenal removed and insulin withdrawn	3	12.0	9.0	6.0	4–8
Group III	A. Depancreatized. Adrenals intact	10	0.0	16.0	4.6	3–8
	B. Depancreatized. Adrenals denervated	5	3.0	23.0	6.6	3–12
	C. Depancreatized. Adrenals demedullated	5	8.0	21.0	5.0	2–7
	D. Pancreas and second adrenal removed	4	3.0	20.0	16.0	14–28
Group IV	Adrenalectomized, then depancreatized	4	2.0	20.0	18.0	11–25
Group V	Hypophysectomized, then depancreatized	6	5.0	40.0	48.0	35–85

those reported by other workers who have followed similar procedures on the dog (3–8).

In Table I, groups III B and III C, will be seen the effects of these operations upon the survival of depancreatized cats. It will be apparent that the slight increase in the survival period cannot be taken as evidence in support of the experiments of Barnes *et al.*, particularly as Table II shows that the severity of the diabetes as judged by other criteria was in no way abated.

When we consider the effects of a total adrenalectomy upon the

survival of depancreatized cats we are drawn to the conclusion that it is the loss of the adrenal cortex rather than the medulla that is responsible for the amelioration of the diabetes observed.

Our first adrenalectomized-depancreatized cats, Table I, group I B, which were prepared by removal of a pancreatic transplant along with the second adrenal did not have a markedly increased period of survival. Nevertheless no animal died in less than 6 days and 2 survived for 12 days. It was, however, the striking changes in the glycosuria and ketonuria that led us to prepare animals whose condition had not been lowered by extensive preliminary operations.

In 10 animals the whole of the pancreas and the remaining adrenal were removed at the same time. 3 were sacrificed in good health for liver glycogen and fat determinations 5 days later. 2 animals developed serious wound infections and died in 7 and 9 days respectively, while one animal relapsed into diabetic coma and died, subsequent examination revealing a large accessory adrenal cortical body (No. 1-66).

The remaining 4 animals (cited in Table I, group III D and Table II F) had no complications of this character and it is apparent that in them the survival period has been significantly increased.

One animal, No. 1-69, was killed 28 days after pancreatectomy, another, No. 1-67, died 17 days later, while the remaining 2, Nos. 2-10 and 2-14, died 2 weeks after extirpation of the pancreas.

The last group of adrenalectomized-depancreatized cats (Table I, group IV, and Table II G) are those prepared by removal of the pancreas from adrenalectomized animals that had been maintained in good health upon cortical extracts. Cat 2-20 was maintained for 28 days after adrenalectomy upon a cortical extract.[2] Following pancreatectomy it survived for a further 19 days.[3] Cat 2-25 was treated with eschatin for 14 days before pancreatectomy and survived 11 days after pancreatectomy. Cat 2-36 was treated for 7 days with an extract,[4] then depancreatized. It survived 16 days. Finally, cat 2-39 was in good health 9 days after adrenalectomy and treatment with an extract prepared by us according to the method of Swingle and Pfiffner. When depancreatized it lived for another 25 days.

In view of the survival periods of the animals in groups III D and IV we are of the opinion that total adrenalectomy previous to or

[2] This extract was supplied by Dr. J. J. Pfiffner.

[3] This cat was found at autopsy to possess a small cortical body 1 mm. in diameter.

[4] This extract was kindly supplied by Dr. S. W. Britton.

synchronous with total pancreatectomy increases the survival period of the cat over that usually observed in the animal depancreatized with adrenal cortical tissue left intact.

As in the dog, hypophysectomy previous to pancreatectomy is followed by very long survival periods. The average of our 6 Houssay cats (Table I, group V and Table II E) was 48 days, one animal surviving almost 3 months. These periods far exceed those found in the adrenalectomized-depancreatized cats, but factors at present not understood may be operating to shorten life in the latter.

Behavior and Mode of Death.—It has long been recognized that the usual mode of death of depancreatized dogs is in diabetic coma, attended by acidosis. This is not an invariable rule, since greatly emaciated animals may die of inanition without coma, not however, without exhibiting gross glycosuria and some ketonuria prior to death.

It may be categorically stated that none of our adrenalectomized-depancreatized nor hypophysectomized-depancreatized cats spontaneously developed diabetic acidosis, ketosis or coma, in striking contrast to the depancreatized animals in which this sequence of events is an almost invariable occurrence. Thus, out of 29 cats deprived of insulin, either by pancreatectomy or withdrawal of this substance subsequent to pancreatectomy, 26 relapsed into coma before death, of the remaining animals the death of one could probably be attributed to peritonitis following the removal of a pancreatic transplant, while the other 2 died of inanition 8 to 12 days after operation, not, however, without first exhibiting the same perversion of metabolism as was observed in the greater number.

This immunity to acidosis and ketosis that is so characteristic of the doubly operated animals is reflected in their improved appetite and behavior. The depancreatized cat rarely eats except during the first 24 or 48 hours after operation. As soon as ketone bodies appear in the urine food is refused and the animal gradually becomes apathetic and slips into a comatose condition. On the other hand, the appetite of the doubly operated animals is at first very poor, and all food may be refused for several days. In good preparations the ingestion of food is then resumed and, in fact, they often exhibit the voracious appetite seen in depancreatized animals maintained upon insulin. This is particularly so in the case of the hypophysectomized-depancreatized cats; but the adrenalectomized-depancreatized group as a rule have rather poor appetites.

All the doubly operated animals lost weight even when consuming amounts of food adequate to maintain normal animals (Table I). The degree of weight loss was proportional to the length of survival and in the case of the hypophysectomized-depancreatized animals may reach 40 per cent of the initial value before death occurs. These animals ultimately succumb to inanition partly as a result of the mild diabetes that persists and partly as a result of the factors introduced by the loss of the external pancreatic secretion.

In the adrenalectomized-depancreatized animals death has frequently occurred suddenly, often when the animals had lost but little weight and were eating well. The high incidence of hypoglycemic attacks in these animals has led us to suspect this factor as the cause of these often unexpected demises. Our reasons for supposing that these hypoglycemic episodes were not entirely due to a deficiency of the water and salt metabolism hormone of the adrenal cortex will be considered later.

Glycosuria, Nitrogen and Ketone Body Excretion.—(a) During Fasting.—In the fasting depancreatized animal the blood sugar remains elevated while glucose continues to appear in large amounts in the urine. In addition, the urinary nitrogen excretion is elevated two or threefold above that found in the normal fasting animal. Even more characteristic is the presence in the urine of acetone bodies, often in large amounts. That these phenomena are found in the fasting depancreatized cat is demonstrated in Table II B, C and D. It will be observed that the glucose excretion is about 3 gm. per kilo of body weight a day, while the urinary nitrogen is doubled or trebled as compared to that found in normal cats during similar fasting periods. The ketonuria is very marked, while the D/N ratio, although by no means constant from animal to animal, is set at a high level.

The animals in Table II C and D leave no doubt that the extirpation of the adrenal medulla or paralysis of its secretion by nerve section does not prevent the appearance of the characteristic high levels of glucose, nitrogen and ketone body excretion, following pancreatectomy.

Cat 1-65, Fig. 1, illustrates the difference between the effects of adrenal denervation and total adrenalectomy upon pancreatic diabetes in the same animal

The right adrenal was removed on Oct. 5, 1934, and the left adrenal denervated on Oct. 24. Total pancreatectomy was performed on Oct. 31 and no insulin was administered. In spite of fasting the glycosuria promptly rose to a high level

TABLE II

Glucose, Nitrogen and Acetone Body Excretion of Various Cats during Fasting

Cat No.	Days after pancreatectomy	Glucose	Nitrogen	Acetone	D/N
		A. Normal			
	days	*g/k/d**	*g/k/d**	*mg/k/d**	
1-77	Fasting 5	—	0.6	9	—
1-85	" 7	—	0.6	—	—
1-86	" 7	—	0.4	—	—
2-03	" 7	—	0.7	10	—
2-04	" 7	—	0.6	15	—
2-05	" 6	—	0.8	23	—
2-06	" 6	—	0.7	—	—
Average			0.6	15	—
		B. Depancreatized—Adrenals Intact			
1-73	1st–3rd	3.0	1.1	28	2.8
1-76	1st–3rd	3.7	1.6	102	2.3
1-87	1st–3rd	2.2	1.0	85	2.2
2-00	1st–5th	2.9	1.4	275	2.1
2-47	1st–4th	4.2	1.7	174	2.4
Average		3.2	1.4	133	2.4
		C. Depancreatized—Adrenals Denervated			
1-95	1st–9th	3.4	1.1	64	3.0
2-03	1st–12th	2.3	1.3	84	1.9
2-05	1st–7th	3.0	1.1	220	2.8
2-07	1st–3rd	3.4	1.0	230	3.5
Average		3.0	1.1	150	2.8
		D. Depancreatized—Adrenal Medullae Removed			
1-98	1st–6th	2.7	1.2	125	2.2
1-99	1st–3rd	2.3	1.2	100	1.9
2-01	1st–6th	2.5	1.3	83	1.9
Average		2.5	1.2	103	2.0
		E. Hypophysectomized and Depancreatized			
1-40	1st–4th	1.3	0.9	7	1.6
1-83	1st–6th	0.8	0.6	5	1.4
2-22	32nd–34th	0.3	0.5	—	0.6
2-26	1st–3rd	0.5	0.6	12	0.8
Average		0.7	0.7	8	1.1
		F. Simultaneous Adrenalectomy and Pancreatectomy			
1-67	2nd–4th	0.7	0.6	15	1.2
1-69	4th–6th	0.1	0.4	6	0.3
2-10	1st–4th	0.1	0.4	2	0.3
2-14	1st and 2nd	0.3	0.9	—	0.3
2-15	1st–3rd	0.4	0.5	—	0.8
2-21	1st–3rd	0.0	0.5	20	—
2-25A	1st–4th	0.5	0.7	12	0.7
Average		0.3	0.6	11	0.5
		G. Adrenalectomized and Then Depancreatized			
2-20	1st–3rd	1.1	0.7	11	1.6
2-25	2nd–4th	0.3	0.5	7	0.6
2-36	1st–4th	0.8	0.7	9	1.1
2-39	2nd–4th	0.4	0.7	9	0.6
Average		0.7	0.7	9	1.0

* Signifies grams or milligrams per kilo of body weight a day.

and 24 hours later marked acetonuria was present. The institution of insulin therapy and dietary control promptly led to a diminution in the glycosuria and a disappearance of the acetonuria. On Nov. 7 the left adrenal was removed and insulin withdrawn. During the ensuing week, glycosuria was very slight and acetonuria did not appear. 8 cc. of a potent cortical extract prepared by Swingle and Pfiffner's method were injected daily.

As Houssay and Biasotti (23) have pointed out, the most striking effects of hypophysectomy upon pancreatic diabetes are the changes observed in the endogenous protein metabolism. In confirmation of this Table II E shows that not only is the glucose excretion greatly

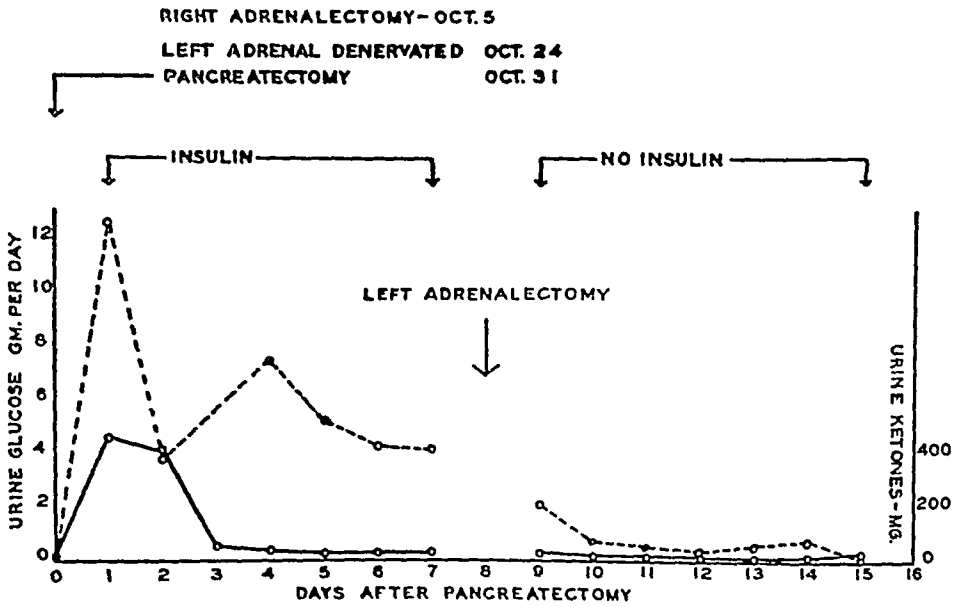


FIG. 1. Cat 1-65. The negative effect of adrenal denervation and the marked effects of total adrenalectomy upon pancreatic diabetes. Dotted line represents urine glucose, the full line urine ketone bodies. For details see text.

reduced but the nitrogen excretion is restored to the normal fasting level in the hypophysectomized-depancreatized cat. Even more characteristic is the absence of ketosis and acidosis as is indicated by the slight urinary excretion of the ketone bodies. The removal of the main factors participating in the development of diabetic coma must account in large part for the increased survival of the animals.

Table II F and G shows that the effects of adrenalectomy upon the fasting diabetic metabolism are identical with those of hypophysectomy. There is the same marked reduction in the urinary excretion of glucose, nitrogen and ketone bodies, and it is difficult to avoid

the conclusion that both these procedures are producing their effects by interference with the same phases of the diabetic metabolism.

(*b*) *On a Constant Diet.*—Table III contains the results of studies made upon (*a*) normal, (*b*) hypophysectomized-depancreatized, and (*c*) adrenalectomized-depancreatized cats when fed for several days upon a meat diet of 100 gm. of canned salmon and 40 gm. of raw pancreas a day. The glucose excretion of the well fed hypophysectomized-depancreatized cats is still slightly below that of the fasting depan-

TABLE III

Glucose, Nitrogen and Ketone Excretion while Eating 40 Gm. Pancreas and 100 Gm. Salmon Daily

Cat No.	Days after pancreatectomy	Glucose	Nitrogen	Ketones	D/N
		A. Normal			
	days	*g/k/d*	*g/k/d*	*mg/k/d*	
1-41	16 day period	—	1.5	13	—
1-68	8 " "	—	1.7	15	—
Average.....			1.60	14	
		B. Hypophysectomized and Depancreatized			
1-40	13th-32rd	1.7	1.0	7	1.7
1-60	4th-20th	2.3	1.3	7	1.8
2-22	21st-31st	3.2	1.6	12	2.0
2-30	5th-14th	1.6	1.3	—	1.2
Average.....		2.20	1.30	9	1.9
		C. Adrenalectomized and Depancreatized			
1-67	6th-16th	1.6	1.2	6	1.3
2-10	5th-13th	0.5	1.0	4	0.5
Average.....		1.05	1.10	5	0.9

creatized animals, while the nitrogen excretion is at about the same level.

Blood Sugar Level and Hypoglycemic Episodes.—In the depancreatized cat the fasting blood sugar is almost invariably greater than 200 mg. per cent. In the terminal stages of coma it rises to exceedingly high levels, values exceeding 500 mg. per cent being not uncommon.

Houssay and Biasotti (15) have called attention to the fluctuating character of the blood sugar level in hypophysectomized-depancreatized dogs and have noted that actual hypoglycemic episodes

occasionally occur. Our own experience with similarly operated cats in general supports these findings. When such cats are fasted, not only may the glycosuria disappear, but the blood sugar may fall to such low levels that death from the resulting hypoglycemia occurs. This was the case in 2 cats in our series. In well fed animals wide fluctuations in the fasting level also occur from day to day, even when the food intake remains constant. Curiously enough, the amount of glycosuria of such an animal with constant dietary intake is much more uniform, and it would appear that the fasting blood sugar level is subject to marked variation depending upon the length of time elapsing since the last meal. It has been recognized that the fasting hypophysectomized animal is often subject to a fatal hypoglycemia, but it is remarkable that even the complete absence of the pancreas does not always protect it against this circumstance.

The untreated adrenalectomized cat is very prone to exhibit a lowered blood sugar level even before the grosser signs of insufficiency are present (24). Terminal hypoglycemic symptoms are of common occurrence in such animals. The usual view that has been taken is that the marked reduction in the blood sugar level is associated with the absence of the cortical hormone, together with the refusal of food that invariably occurs a few days before death. Furthermore, it has been noted that the injection of glucose, although increasing the blood sugar and effecting some temporary improvement, does not prolong the lives of such animals more than a few hours. Of particular interest in this respect are the recent observations of Harrop, Soffer, Nicholson and Strauss (25). These authors report that adrenalectomized dogs maintained in excellent health by the injection of the cortical hormone or by the use of ample sodium chloride and bicarbonate feedings relapse into severe hypoglycemia when fasted for a few days. Such animals are at once restored to good health by glucose injections. It is obvious that these experiments raise the question as to whether the hypoglycemia commonly observed in adrenal insufficiency is to be attributed to the lack of the cortical hormone controlling water and salt metabolism or whether it is to be looked upon as evidence of an associated disturbance in carbohydrate metabolism brought about by removal of the adrenal glands, and not compensated for by the injection of amounts of extract capable of controlling other manifestations of adrenal insufficiency.

These remarks are prompted by our observation that the adrenalectomized-depancreatized cat is even more prone to hypoglycemic episodes than is the hypophysectomized-depancreatized animal. Thus, out of 18 adrenalectomized-depancreatized cats, 5 developed such severe hypoglycemia that glucose injection was necessary to save their lives. It should also be emphasized that these animals lived for several days after such hypoglycemic episodes, which is evidence that the low blood sugar level in these instances was not associated with severe adrenal insufficiency, not only because cortical extract was being injected, but also by the established fact that glucose only temporarily resuscitates animals suffering from severe adrenal insufficiency. An example will illustrate this:

Protocol of Cat 2-39

Right adrenalectomy May 16th. Weight 2.5 kilos.
Left adrenalectomy May 25th. Weight 2.5 kilos.
Maintained in excellent condition for 9 days by 4 cc. daily of cortical extract.
Pancreatectomy June 3rd. Weight 2.5 kilos.
Killed June 28th. Weight 1.7 kilos. Survival 25 days.

Date	Meat eaten	Urine glucose	Blood sugar	Cortical extract	Notes
	gm.	*gm.*	*mg. per cent*	*cc.*	
June 17	65	1.8	226	6	
" 18	0	0.9	13	6	Very weak, relieved at once by 3 gm. glucose
" 19	90	3.6	246	6	
" 20	60	1.0	212	6	
" 21	20	0.0	173	10	
" 22	90	1.4	104	10	
" 23	60	2.5	—*	10	Very weak, relieved at once by 3 gm. glucose
" 24	115	2.9	118	10	
" 25	98	1.7	28	10	Mild symptoms relieved by 50 cc. milk
" 26	50	Lost	—	10	
" 27	0	Trace	63	10	
" 28	Animal killed				

Terminal serum urea nitrogen 66 mg. per cent. Terminal serum CO_2 combining power 41 volumes per cent. Liver glycogen 1.0 per cent. Liver fatty acids 9.4 per cent.

* Too weak to read.

Carbohydrate Tolerance. (*a*) *Injected Glucose.*—Houssay and Biasotti (23), Biasotti (26) and Barnes and Regan (27) have stated that in contrast to the depancreatized animal the ingestion of glucose by the hypophysectomized-depancreatized dog is followed by a lowered blood sugar curve and that considerable quantities of the ingested glucose are retained.

TABLE IV

Glucose Tolerance Tests

Cat No.	Days after pancreatectomy	Glucose	Method of administration	Blood sugar						Notes
				Before	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	
A. Hypophysectomized—Depancreatized										
	days	*gm.*		*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	
1-57	21	1.5	Intraperitoneally	37	149	138	118	—	—	In shock at onset
2-22	9	3.0	"	206	326	340	318	—	328	
2-22	50	3.0	"	140	282	308	380	324	312	
2-27	4	3.0	"	210	394	416	388	392	372	
B. Adrenalectomized—Depancreatized										
8-6	2	3.0	Mouth	68	—	152	—	—	—	
8-6	8	3.0	"	32	88	86	90	—	—	In shock
1-17	1	3.0	"	138	—	—	340	—	—	
1-09	2	3.0	"	104	171	147	115	—	—	
9-8	7	2.5	Intraperitoneally	18	—	—	—	—	134	In shock
1-17	4	3.0	"	28	—	—	—	—	104	" "
1-09	4	3.0	"	42	226	225	232	219	217	
2-10	14	3.0	"	30	—	133	—	—	—	In shock
2-15	4	3.0	"	184	344	326	298	—	272	
2-21	4	3.0	"	240	272	300	332	340	314	
2-39	15	3.0	"	13	—	119	111	—	123	In shock

Table IV contains the results of our experiments with glucose feeding or intraperitoneal injection into hypophysectomized-depancreatized and adrenalectomized-depancreatized cats.

As will be observed, in six instances the glucose was administered as the animals were exhibiting various degrees of hypoglycemic shock. Since this invariably occurred as a result of fasting, they represent the effect of glucose upon the blood sugar under these conditions. The remaining figures were obtained by administering glucose to the animals after an overnight fast.

It will be apparent that when the initial blood sugar level in the animals that we have tested is below 40 mg. per cent, the response to glucose is essentially normal, whereas if the blood sugar level is elevated above the normal level, a typically diabetic response is obtained. In interpreting this condition it should be noted that the animals with low blood sugar levels were in varying degrees of prostration, often with subnormal body temperatures. As a consequence, it is possible that the absorption of the administered glucose was delayed. Since the condition of such animals was almost at once improved by the glucose, and as they did not subsequently excrete it even when observed for many hours, it is difficult to avoid the conclusion that when these hypoglycemic episodes occur the utilization of considerable quantities of ingested glucose is possible even in the complete absence of the pancreas.

Quite a different picture is presented when glucose is administered to doubly operated animals in good health and with elevated initial blood sugars. From an observation of a considerable number of such animals we are of the opinion that when the fasting blood sugar is elevated these animals are clinically in their best condition, and at this time the administration of glucose gives a blood sugar response indistinguishable from that found in totally depancreatized animals.

(*b*) *Addition of Glucose to a Constant Diet.*—From the results in Table IV it would be expected that the addition of glucose to the diet of animals in good health would result in its almost quantitative excretion. This has been carried out in the case of two hypophysectomized-depancreatized cats, both of which survived for over 50 days after pancreatectomy and ate 100 gm. of canned salmon and 40 gm. of pancreas daily. After a suitable fore period 3 gm. of glucose were added to each of the two daily meals for 3 days. This was readily consumed, but, in both cases practically a quantitative excretion was observed. Since the animals were excreting 4–7 gm. of glucose daily during the fore period, this result is not surprising.

(*c*) *Utilization of the Glucose Derived from a Meat Diet.*—It has long been recognized that when a completely phloridzinized or depancreatized dog is fed upon a protein and fat diet, a constant ratio between urine glucose and nitrogen is established. In the depancreatized cat, before the development of severe acidosis, this ratio is 2.8.

Houssay and Biasotti (29) have demonstrated that the urinary D/N ratio of the hypophysectomized-depancreatized dog is much less than the values established for the totally depancreatized animal. A study of these ratios in our cats indicates that these low values are also found in the adrenalectomized-depancreatized cats (Table II).

If we know the amount of urine nitrogen excreted and if we assume that in the doubly operated cats the same proportion of protein is converted into glucose, we are in a position to calculate the quantity

TABLE V

Utilization of Glucose by Hypophysectomized-Depancreatized and Adrenalectomized-Depancreatized Cats as Calculated from Urinary Glucose and Nitrogen Excretion

Cat No.	Food daily	Period	Average urine nitrogen per day	Average urine glucose per day	Available glucose Urine $N_2 \times 2.8$	Glucose retained	Glucose retained
	A. Hypophysectomized—Depancreatized						
		days	*gm.*	*gm.*	*gm.*	*per cent*	*g/k/d*
2-22	40 gm. pancreas, 100 gm. salmon	11	3.4	6.7	9.5	29.5	1.3
2-30	" "	10	3.5	4.3	9.8	56.1	2.1
1-40	" "	20	2.8	4.8	7.8	38.5	1.1
1-60	" "	17	3.2	5.8	9.0	35.6	1.3
1-83	Fasting	6	2.1	2.9	5.9	51.0	0.8
2-22	"	3	0.9	0.5	2.5	80.0	1.0
2-26	"	3	2.2	1.8	6.2	71.0	1.3
	B. Adrenalectomized—Depancreatized						
1-67	40 gm. pancreas, 100 gm. salmon	11	2.3	3.0	6.5	54.0	1.8
2-10	" "	9	3.7	1.8	10.4	82.6	2.3
2-25A	Fasting	4	1.7	1.4	4.8	71.0	1.3
2-36	"	4	1.9	2.0	5.3	62.4	1.2

of glucose formed in the body by the metabolism of protein. This figure may then be compared with the actual glucose excretion. Any difference between the two should represent the glucose derived from protein that has been retained. Similar calculations can also be made for the fasting animal.

Table V contains the results of calculations made in this manner. It is apparent that in these doubly operated animals a large proportion of the glucose derived from protein is retained. In some animals this proportion may be over 80 per cent of the calculated amount. Never-

theless the actual amounts of carbohydrate utilized are very small (about 1–2 gm. per kilo a day) and it would appear that the capacity of the tissues of these animals to assimilate glucose derived from protein, although perhaps superior to that of the depancreatized group, nevertheless remains at a low level.

In conclusion, in view of these three different types of experiments, we are of the opinion that the carbohydrate tolerance of the depancreatized cat is but little improved by the removal of either the hypophysis or adrenals. We except from this statement the curious results obtained with animals in the hypoglycemic state in which it is obvious that other factors are operating.

The Influence of the Water and Salt Hormone of the Adrenal Cortex upon the Diabetes of Adrenalectomized-Depancreatized Cats

One question remains: To what extent are the effects of adrenalectomy upon pancreatic diabetes due to the loss of the cortical hormone controlling water and salt metabolism? Since the removal of this hormone is followed by marked metabolic disturbances it might be argued that the effects upon pancreatic diabetes are merely secondary to the deficiency of this hormone.

It has been demonstrated (28) that the cachexia of hypophysectomized rats is not relieved by cortical extracts adequate to maintain life in adrenalectomized dogs. Furthermore, the hypophysectomized dog does not exhibit any of the characteristic features of cortical insufficiency, yet there is no doubt as to the different character of experimental pancreatic diabetes in such animals. In addition, we have found that the glycosuria or ketonuria of hypophysectomized-depancreatized cats is unaffected by the injection of 100–300 dog units of the cortical extracts at our disposal.

Although these experiments demonstrate that hypophysectomy brings about an amelioration of pancreatic diabetes even when ample supplies of cortical hormone are present, they do not answer the possibility that a deficient supply of this substance may be responsible for the apparently equally effective results of adrenalectomy.

The following observations speak against this possibility.

(a) As Table VII shows, the effects of adrenalectomy upon pancreatic diabetes are well marked during the first 48 hours after simultaneous removal of both

endocrine organs. Since the full effects of adrenal insufficiency are not manife for several days it is considered unlikely that any loss of the water and sa hormone would be reflected upon the carbohydrate metabolism at such a early date.

(*b*) All the adrenalectomized-depancreatized animals received large amoun of this cortical hormone in terms of dog units daily. We have already point out the necessity for these large doses in the cat, and while it is true that some our earlier animals were probably inadequately treated this was not the case f all of them. Thus, while the average daily dose given to the adrenalectomize

TABLE VI

Type of animals	No. of animals		Cortical extract	Total base	Chlorides	CO_2 combining power	Urea nitrogen	Glucose	Cholesterol	Dry weight
			*du/k/d**	*m.-eq.*	*m.-eq.*	*vol. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*gm. per ce*
Normal	5	Average...	—	159	121	43	28	108	80	7.3
		Range.....	—	156–162	117–126	39–48	24–35	95–115	38–115	7.0–7.
Depancreatized	11	Average...	—	147	98	24	81	592	132	8.9
		Range.....	—	127–159	78–118	15–38	17–150	338–1050	57–162	7.3–11
Adrenalectomized and showing marked symptoms	2	Average...	None	135	104	26	74	79	135	9.0
		Range.....	—							
Adrenalectomized and depancreatized	12	Average...	28	150	108	37	53	179	101	8.5
		Range.....	18–47	135–165	98–122	21–50	30–84	30–308	42–200	7.2–9.
Hypophysectomized and depancreatized	4	Average...	—	156	119	45	45	207	132	7.6
		Range.....	—	154–159	114–122	31–50	29–52	40–320	69–197	6.1–10

All the analyses in this table have been carried out by Dr. F. W. Sunderman this department.

* Signifies dog units per kilo a day.

depancreatized cats in Table I, groups I and II, was only 18 dog units (rang 8–37), we can detect no difference (except length of survival) in the amelioratio of the diabetes between these animals and those in groups III and IV which re ceived an average daily dose of 28 dog units (range 18–47) of cortical extract prepared by the method of Swingle and Pfiffner.

(*c*) Animals suffering from a deficiency of the water and salt controlling hor mone of the adrenal cortex exhibit characteristic alterations in the electrolyt pattern of their serum.

In Table VI we have averaged the electrolyte values of the serum of cats tha have undergone various operations. In all cases the blood was collected unde

oil from the anesthetized animal after the survival periods indicated in Table I. This period was short in the depancreatized or adrenalectomized animals and relatively long in the adrenalectomized and depancreatized or hypophysectomized and depancreatized groups.

The figures demonstrate that in the hypophysectomized-depancreatized group there is but little alteration in the electrolyte pattern, again emphasizing the point that an inadequate supply of cortical extract is not a prerequisite for the alleviation of the diabetes observed in hypophysectomized animals. However, in the adrenalectomized-depancreatized cats in spite of the large amounts of cortical extract given, there is on the average a small diminution in the total base

TABLE VII

The Glucose, Nitrogen and Acetone Body Excretion of Adrenalectomized-Depancreatized Cats Receiving Large Amounts of Cortical Extract

	No. 2-56, adrenalectomized				No. 2-58, adrenalectomized				
Days after pancreatectomy	1	2	3	4	1	2	3	4	5
Cortical extract, *dog units*	1040	520	650	650	1040	260	260	650	650
Meat eaten, *gm.*	20	85	70	110	0	0	25	55	100
Glucose, *gm. per kilo*	0.5	1.3	1.1	1.0	1.5	0.7	0.5	1.1	1.8
Nitrogen, *gm. per kilo*	1.7	1.1	1.0	1.5	0.7	0.7	0.5	0.8	1.3
Acetone bodies, *mg. per kilo*	0	17	15	11	4	5	3	4	9
D/N	0.3	1.2	1.1	0.7	2.1	1.0	1.0	1.4	1.4
	No. 2-61, adrenalectomized				No. 2-47, adrenals intact				
Days after pancreatectomy	1	2	3	4	1	2	3	4	5
Cortical extract, *dog units*	1300	910	650	650	0	0	0	0	
Meat eaten, *gm.*	55	40	40	50	0	0	0	0	
Glucose, *gm. per kilo*	1.2	0.6	0.8	0.8	3.8	3.8	4.2	1.6	Diet
Nitrogen, *gm. per kilo*	1.4	0.6	0.6	0.7	1.7	1.7	1.5	0.6	
Acetone bodies	0	9	10	4	148	148	251	25	
D/N	0.8	1.0	1.3	1.1	2.2	2.2	2.8	2.7	

with a somewhat larger decrease in the serum chloride, together with an increase in the blood urea and some degree of hemoconcentration. It is apparent that towards the end of their survival certain animals have developed some degree of insufficiency, others, to the contrary, have retained a practically normal serum pattern. As we have mentioned before, we have been unable to differentiate these animals by the degree of glucose or acetone body excretion.

(*d*) One of the most cogent arguments against a deficiency of the water and salt hormone as the reason for the amelioration of diabetes in adrenalectomized cats is the animals in Table II G. These were maintained in good health by cortical extracts for periods from 7–28 days before pancreatectomy. The amelioration of the diabetes that followed was of the same extent as that observed in

animals in which the adrenalectomy and pancreatectomy were performed in one stage.

Since the above was written we have recently studied 3 additional adrenalectomized-depancreatized cats to which large amounts of a very potent cortical extract[5] were administered. One of these animals (No. 2-61) was adrenalectomized and maintained in good health for a month before pancreatectomy. The other 2 animals (Nos. 2-56 and 2-58) had the remaining adrenal and all the pancreas removed at one operation. The daily glycosuria, nitrogen excretion and ketonuria along with that of a depancreatized cat are given in Table VII. The degree of amelioration of the diabetes was as great as that previously observed, although one animal received three times the amount of cortical extract that had been sufficient to maintain it before pancreatectomy.

DISCUSSION

The data presented indicate that the removal of the hypophysis or adrenal glands modifies in all its aspects the sequence of events that usually follows total pancreatectomy in the cat. Not only is the survival of these animals significantly increased, but death, when it ultimately occurs, is not preceded by a period of severe acidosis and ketosis. Even more significant is the fact that during life the characteristic and extreme perversions of metabolism are greatly modified. In brief, either hypophysectomy or adrenalectomy convert the diabetes from a severe, rapidly fatal form into one of moderate degree in which inanition rather than acidosis is the ultimate cause of death.

These modifications are expressed by:

1. The virtual abolition of ketosis and acidosis. It is the removal of these factors that is undoubtedly responsible for the prolongation of life. Of even greater importance is the manner in which this alteration is produced. There are at least three possibilities: (*a*) The rate and amount of fat mobilized into the liver and there metabolized may be greatly reduced. This is supported by some preliminary observations in which we have found that the usual intense fatty infiltration of the liver of depancreatized cats does not occur in the same length of time in the doubly operated animals. Nevertheless, over longer periods some fatty infiltration does take place and at autopsy in animals surviving for long periods all the body stores of fat are utilized. (*b*) In animals without pituitary or adrenals the complete oxidation

[5] This extract was prepared by Dr. Cartland of the Upjohn Co.

of acetone bodies may be possible, particularly if they are not delivered to the tissues in the usual excessive amounts. Along classical lines it might be argued that sufficient oxidation of carbohydrates has been resumed to effect this utilization. Our present findings, however, indicate that the utilization of carbohydrate is but little improved by these procedures. (*c*) Finally, it is possible in these doubly operated animals that fat metabolism follows a pathway not involving the formation of these ketone bodies.

2. The excessive conversion of the body protein is greatly reduced in the doubly operated animals. As a result, during fasting, the glycosuria falls to very low levels and is associated with a corresponding reduction in nitrogen excretion. This protein sparing effect can again hardly be attributed to the resumption of sufficient carbohydrate oxidation since all our present evidence is to the contrary.

3. This persistence of a markedly impaired carbohydrate metabolism together with a reduction in the intensity of protein and fat metabolism indicates that the probable effect of hypophysectomy or adrenalectomy upon pancreatic diabetes is a diminution in the production of glucose and ketone bodies, rather than a restoration of carbohydrate oxidation. All the evidence indicates that normal carbohydrate oxidation is impossible in the complete absence of insulin.

It is generally agreed that it is the removal of the anterior portion of the hypophysis that is responsible for the effects produced by a total hypophysectomy. The evidence we have presented here, together with the results previously reported, would seem to establish the fact that the removal or paralysis of the secretion of the adrenal medulla is without effect upon the results of a total pancreatectomy. The effects of adrenalectomy must therefore be due to the ablation of the cortical portion of the gland. It appears to us that removal of the adrenal cortex may produce an amelioration of pancreatic diabetes in three possible ways.

1. In spite of the experiments we have cited in Table VII, the water and salt hormone of the cortex was not supplied in sufficient quantity for the diabetes to develop in its usual manner when the pancreas was removed (11).

2. Removal of all cortical tissue might produce alterations in the function of the anterior pituitary of such a nature that its diabetogenic

activity is no longer exerted. In other words, a functional suppression of this organ is brought about by total adrenalectomy. We are engaged at present in testing this hypothesis.

3. The apparently identical effects of adrenalectomy and hypophysectomy might be related by reason of the trophic control that the anterior pituitary exerts over the adrenal cortex. Thus, following hypophysectomy there occurs a marked atrophy of the adrenal cortex, and it is tempting to speculate as to whether the effects of hypophysectomy upon pancreatic diabetes are not mediated by the ensuing occurrence of cortical atrophy. If this explanation is correct then the adrenal cortex plays an essential part in the increased protein and fat catabolism following pancreatectomy.

It is becoming apparent that the anterior pituitary hormones play an important rôle in those conditions under which gluconeogenesis is increased. Thus it has been found (15) that hypophysectomized dogs rapidly develop hypoglycemia when fasted. Furthermore, if such animals are phloridzinized, the glucose, nitrogen and acetone body excretion is greatly diminished compared to that found in the normal animal (29, 30).

Under similar conditions adrenalectomized animals also exhibit marked deviations from the normal. Thus Harrop *et al.* (25) have demonstrated that adrenalectomized dogs exhibit marked hypoglycemia after a short fast, even though they have continued to receive amounts of either cortical extract or sodium salts sufficient to maintain them in good health when fed. Evans (31) has recently found that when adrenalectomized rats either maintained on salt or cortical extract are phloridzinized the excretion of glucose, nitrogen and acetone bodies is much reduced, again emphasizing the identical effects of hypophysectomy and adrenalectomy in a condition in which gluconeogenesis is particularly concerned.

Furthermore, the curious findings of Evans (32) of the effects of exposure to low oxygen tensions upon the liver glycogen, nitrogen and acetone body excretion of the fasting white rat also support the above views. This author has shown in the normal fasted rat that exposure for 24 hours to an atmosphere containing 10.5 per cent oxygen results in a marked formation of liver glycogen. Since the glycogen content of the remainder of the body remains unchanged, this liver glycogen must have had its origin from non-carbohydrate sources and this is

borne out by the finding of an increased nitrogen excretion under these conditions. Adrenalectomized or hypophysectomized rats when similarly treated show neither the increase in liver glycogen nor the excess nitrogen excretion, while on the other hand, animals in which only adrenal cortical tissue is left intact behave as do normal rats. Finally, the administration of large amounts of cortical extracts containing the hormone controlling water and salt metabolism is without effect in restoring to normal the response to these conditions. The mechanism by which low oxygen tensions produce these effects is unknown, yet one feature of the response to them is apparently a stimulation of gluconeogenesis. As before, this stimulus is ineffective if either the adrenal cortex or anterior pituitary is absent.

SUMMARY

1. The hypophysectomized cat shows an alleviation of the diabetes following pancreatectomy comparable to that previously demonstrated by others in the dog.

2. It is possible by various procedures to remove both adrenals and all the pancreas from cats. Such animals have survived for as long as 4 weeks without the use of insulin, the average survival being 18 days. Daily injections of cortical extract are necessary. By contrast, cats deprived of insulin by similar procedures but with adrenals intact survive only 4–5 days.

3. Adrenalectomized-depancreatized cats show as striking an alleviation of the diabetes as do those hypophysectomized and depancreatized. This is expressed by the markedly decreased glucose, nitrogen and acetone body excretion compared to that found in the depancreatized cat, as well as by the increased survival period.

4. Removal of epinephrine by denervation or demedullation of the adrenals does not protect against pancreatic diabetes in the cat.

5. The carbohydrate tolerance of depancreatized cats is not significantly increased by hypophysectomy or adrenalectomy.

The exceptions to this finding have all been in animals presenting varying degrees of spontaneous hypoglycemia.

6. It is our opinion that the effects of hypophysectomy or adrenalectomy upon pancreatic diabetes are due to diminution of the production of glucose and acetone bodies rather than to the resumption of normal carbohydrate utilization.

together with a general discussion of the investigations on experimental pox, particularly with reference to the relation of pox virus to other viruses and of rabbit pox infection to other pock diseases.

Materials and Methods

Pox Virus.—The pox virus tissues used for inoculation were obtained from rabbits of the consecutive transmission series of the Xy171 strain (1). In this series Berkefeld V filtered or unfiltered emulsions of testicular tissue derived from an acute orchitis were injected intratesticularly and the inocula employed in the present experiments were portions of these emulsions. The tissue was ground with alundum and Locke's solution to concentrations of 10 to 15 per cent by weight. Each emulsion was examined for the presence of ordinary aerobic and anaerobic organisms. The bacterial sterility of filtered emulsions was tested in the usual manner against *B. prodigiosus* (1).

Animals.—Albino mice from the Institute breeding stock and albino rats, guinea pigs, and male rabbits from outside sources were used. The rabbits were of hybrid stock and averaged 4 to 6 months of age.

A Jersey Holstein calf 3½ months of age and 75 kilos in weight was used in one experiment.

Routes of Inoculation and Dosage.—Mice were injected intraperitoneally, subcutaneously, intracerebrally, and by nasal instillation. In the case of certain intraperitoneal injections, 0.05 cc. of a 2 per cent sterile starch emulsion was also injected intracerebrally. Rats and guinea pigs were injected by the intratesticular, intraperitoneal, and nasal routes, and in addition, the intradermal route was used for certain guinea pigs. Inoculation of a calf was accomplished by rubbing 1.0 cc. of unfiltered tissue-virus emulsion on scarified shaved skin areas of the sides of the body.

The dosage for the small animals ranged from 0.01 to 1.0 cc., the latter amount being used for intraperitoneal injections in guinea pigs.

Experiments on Mice

The first experiments on the inoculation with pox virus of species other than the rabbit were carried out early in the course of the work when Berkefeld V filtrates were employed for the routine passage of the virus.

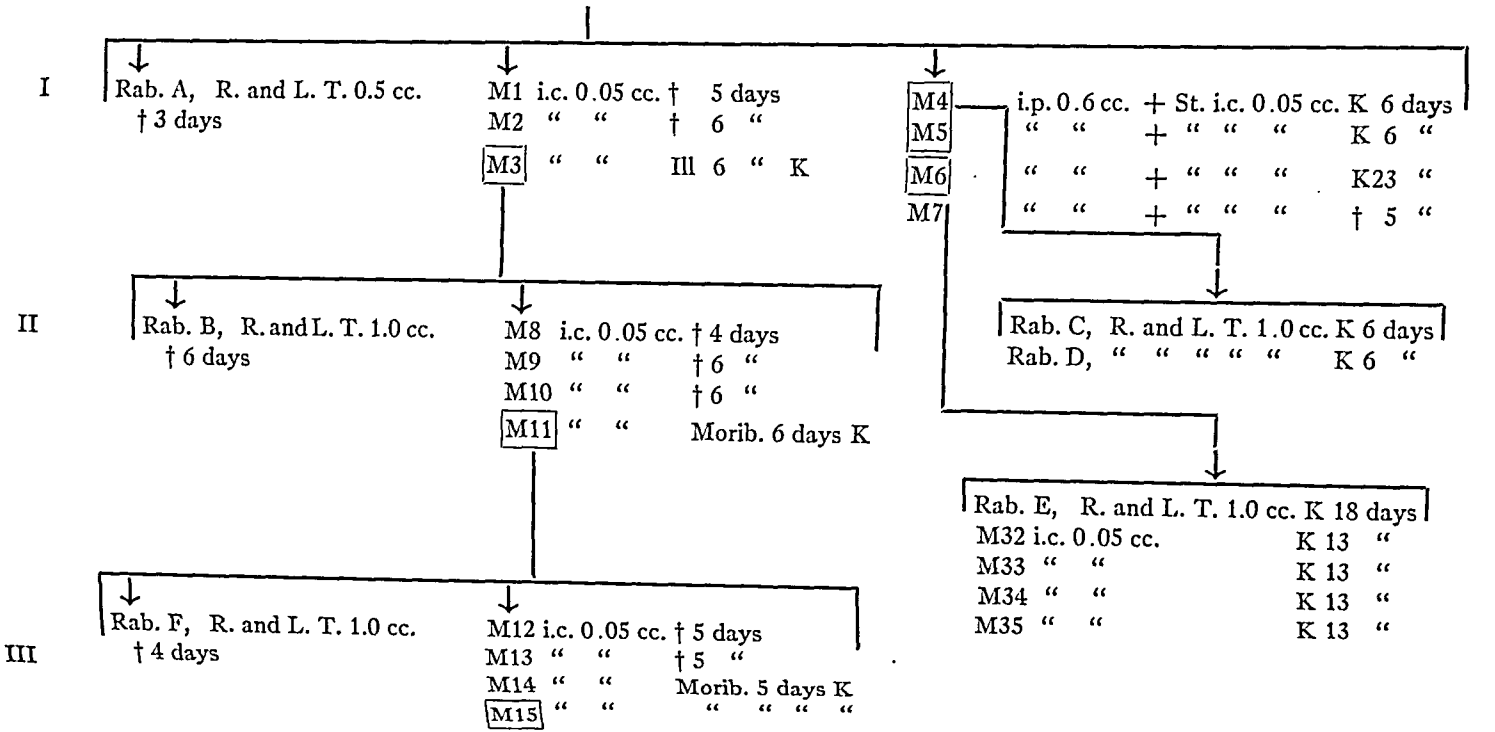
In the first group of 10 mice, a filtrate of testicular tissue from the 2nd serial rabbit passage of the Xy171 strain of virus (1) was injected by the intraperitoneal, subcutaneous, or nasal routes; the dosage ranged from 0.01 to 0.05 cc. A second group of 4 mice were injected intracerebrally with 0.05 cc. doses of a testicular tissue filtrate of the 4th rabbit passage. A third group of 6 mice were injected intraperitoneally with 0.6 cc. of the filtrate of the 6th rabbit passage and intracerebrally with 0.05 cc. of a 2 per cent starch emulsion.

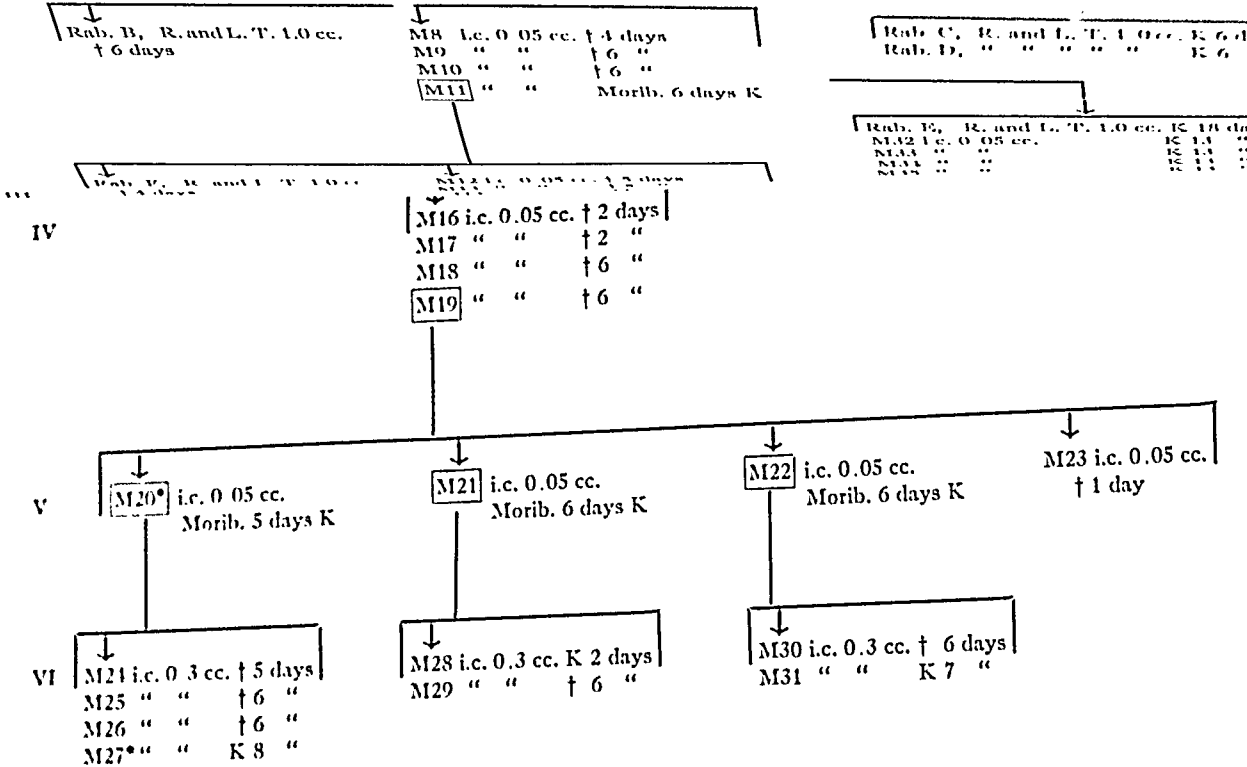
The results of all 3 experiments were negative clinically and at post mortem examination carried out 1 to 4 weeks after injection. Evidence of a successful inoculation in the case of the 3rd experiment, however, was furnished by the results obtained in a rabbit injected with mouse brain.

Two mice were killed 7 days after virus had been injected intraperitoneally and starch emulsion intracerebrally, and a 10 per cent emulsion of the pooled brain tissue was prepared by grinding with alundum in Locke's solution. 1.0 cc. was injected in the testicles of a rabbit; 5 mice were injected intracerebrally with 0.05 cc. doses; 2 mice were injected intraperitoneally with 0.6 cc.; and 2 mice were given 0.6 cc. intraperitoneally and 0.05 cc. starch suspension intracerebrally. The clinical and post mortem results on the mice were negative, but the rabbit developed fever and a marked hemorrhagic orchitis with scrotal edema and was found dead on the 6th day. A Berkefeld V filtrate of testicular tissue from this animal was used to inoculate 2 rabbits with bilateral intratesticular injections of 1.0 and 0.5 cc. respectively. Fever and a pronounced orchitis with scrotal edema developed in both rabbits and in addition, a typical generalized papular cutaneous rash developed on the 8th and 10th days respectively; one rabbit was found dead on the 10th and the other on the 13th day. The clinical results in these rabbits were characteristic of experimental rabbit pox (1, 2) while the post mortem findings were also typical.

Uncertain results were obtained from the injection of 2 rabbits with the brains of 2 other mice of the 3rd series killed at a longer interval after inoculation, that is, a week later than the first 2 mice or 14 days after inoculation. A similar experiment with the brains of mice of the 2nd series killed 7 and 12 days after inoculation had also been inconclusive. The situation was complicated at this stage of the experiments by the fact that after inoculation of certain emulsions of pooled brains had been made, it was found that the emulsions were contaminated. At the time of these particular experiments the retention of virulence of the virus under conditions of refrigeration was not known. In the next experiment, therefore, in which it was planned to carry out a series of brain to brain passages, it was decided to make the inoculations within a week of each other and to use only one brain for each group of animals. In the event that the inoculum was found to be contaminated, it was possible to repeat the experiment with another fresh brain emulsion. A further modification of procedure was the use of an unfiltered emulsion of rabbit tissue virus for the

Pox Rabbit 10th Generation Xy171 Strain Testicular Tissue Emulsion





TEXT-FIG. 1. Results of the serial passage of pox virus in mice. Intracerebral inoculation of unfiltered brain emulsions. i.c. = intracerebral; i.p. = intraperitoneal; T. = intratesticular routes of injection; St. = 2 per cent sterile starch emulsion. † = found dead; K = killed.

* Bacterial contamination of brain of mouse 20. The brain of mouse 27 was sterile.

initial mouse injection instead of the less potent filtrates previously employed.

The results of this experiment were striking. It was found in the first group of mice that the intracerebral injection of unfiltered rabbit tissue virus caused death in from 4 to 6 days, and in addition that this result was repeated in 5 serial passages of unfiltered mouse brain injected intracerebrally (Text-fig. 1).

The series was begun with testicular tissue from a rabbit of the 10th consecutive rabbit passage of the Xy171 strain (1). 3 mice were injected intracerebrally with doses of 0.05 cc.; 4 mice received 0.05 cc. of starch emulsion intracerebrally and 0.6 cc. of virus emulsion intraperitoneally; and a rabbit was inoculated in each testicle with 0.5 cc. As will be seen from the scheme of the experiment given in Text-fig. 1, 2 of the mice inoculated directly into the brain were found dead on the 5th and 6th days; the 3rd mouse was killed on the 6th day and the brain used for the 2nd passage to 4 mice. The results in these latter animals were identical with those in the first group and similar findings were likewise obtained in 4 additional mouse brain passages, that is, a total of 6 mouse generations. In the 6th passage comprising 8 mice the inoculating dose was reduced to 0.03 cc.

In the 2nd and 3rd mouse passages, a rabbit was injected intratesticularly with the mouse brain emulsions. The condition which developed was indistinguishable from the acute fulminating type of infection associated with the intratesticular injection of testicular tissue-virus regularly observed in the rabbit passage series of the virus (1). Fever and a marked hemorrhagic orchitis with scrotal edema developed and the rabbits were found dead on the 4th and 6th days respectively.

Bacteriologic examinations of the mouse brain inocula used for the first 4 serial transfers were negative, but the 5th passage emulsion was contaminated, and consequently two additional sets of inoculations were made with two other 5th generation brains. Cultures of these emulsions were sterile. The results on all three 6th generation groups, however, were similar and furthermore, in the case of the brain of one mouse inoculated with contaminated material and killed 8 days after injection, bacteriologic examination was negative.

The first lot of mice in this experiment inoculated with rabbit tissue-virus included 4 mice which were inoculated intraperitoneally with virus and intracerebrally with starch emulsion (Text-fig. 1). 1 mouse was found dead on the 5th day; 2 were killed on the 6th day and the pooled brains were injected intratesticularly in 2 rabbits; the 4th mouse was killed on the 23rd day and the brain was used for the intratesticular injection of 1 rabbit and the intracerebral injection of 4 mice. Both inocula were bacteriologically sterile. The 2 rabbits injected with the brains inoculated 6 days previously developed fever and an acute hemorrhagic orchitis and scrotal edema typical of experimental fulminating pox infection (1). In the case of the rabbit and the mice injected with mouse brain ob-

tained 23 days after inoculation, the clinical and post mortem results were negative. Although the number of animals is small, the findings suggest that the condition in mice resulting from the intraperitoneal inoculation of pox virus, together with the intracerebral injection of starch, was less severe than that which developed from the intracerebral inoculation of virus alone. In the present experiment, there was only 1 fatality among 4 mice given the double injection while there were 2 deaths among 3 mice injected only with virus and the 3rd mouse was seriously ill when killed. The negative results following the subinoculation of mouse brain 23 days after its injection confirm the previous findings on the uncertainty of recovering virus from this source later than approximately a week after inoculation.

There seems to be no reason to doubt that rabbit pox virus was successfully transmitted to the mouse and was continued in this species by the use of brain emulsions injected intracerebrally, although confirmatory evidence in the form of subinoculations of rabbits is available for only the first 2 passages. The results on the 6 consecutive series of mice were consistent in that a fatal outcome occurred in the great majority of cases and within quite a constant time range. Of 27 mice, 20 were found dead or were critically ill and were killed 5 or 6 days after inoculation; 2 were ill and were killed on the 7th and 8th days respectively; 1 died on the 4th day, 3 on the 2nd, and 1 on the 1st day after inoculation.

In these various experiments none of the inoculated mice developed a disease picture comparable to that of the rabbit with its wide diversity of clinical manifestations. On the other hand, the fatal condition which did develop might be compared to the acute fulminating and rapidly fatal infection of the rabbit associated with a large dosage and the intratesticular route of inoculation such as obtained in the routine serial passage of the virus (1). It is not known how long the virus remains in an active state in the mouse brain, but the results obtained indicate a period of approximately a week after inoculation. Whether the mouse can be infected by routes other than the intracerebral was not sufficiently investigated for any opinion to be formed.

Experiments on Rats

The work on the inoculation of rats with pox virus was limited to 3 experiments, one of which gave results which indicate the susceptibility of this species.

In the first experiment a Berkefeld V filtrate of testicular tissue from a rabbit of the 2nd generation of the Xy171 strain of pox virus was used (1); 2 rats were

injected intratesticularly, 2 intraperitoneally, and 2 by nasal instillation. Doses of 0.1 to 0.2 cc. were employed. In the second experiment unfiltered testicular tissue emulsions of the 10th rabbit generation of the same strain of virus were used; 2 rats were injected intraperitoneally and 2 subcutaneously with doses of 0.5 cc. The results of both experiments were negative in that no clinical evidence of infection developed in any of the animals and the post mortem examinations made 9 to 14 days after injection showed nothing of note.

The third experiment was carried out with testicular tissue from a rabbit of the 15th consecutive passage of the Xy171 strain transmitted by Berkefeld V filtrates. 2 male rats were injected in both testicles with 0.5 cc. of an unfiltered emulsion. There was a slight transitory swelling of the testicles, but no other clinical change was observed. 8 days after inoculation both rats were killed and one testicle of each animal was used for the subinoculation of a rabbit. 10 per cent tissue emulsions were made in the usual manner. Bacteriologic examinations of the emulsion were negative. Each emulsion, unfiltered, was injected intratesticularly in 0.5 cc. doses. Similar results were obtained in both subinoculated animals. Fever and a marked acute hemorrhagic orchitis with accompanying scrotal edema developed and the animals were found dead on the 5th and 7th days respectively. The findings were typical of the acute fulminating type of pox infection regularly produced by the serial transmission of virus in rabbits by means of intratesticular injections of testicular tissue emulsions (1).

The results on these rabbits indicate that pox virus may survive 8 days in the rat and apparently without an attenuation of virulence. It is not known how frequently such results would be obtained nor whether the virus can be serially transmitted from rat to rat. It would seem likely, however, that serial transmission could be accomplished by means of testicle to testicle passage carried out at approximately weekly intervals.

Experiments on Guinea Pigs

The results of experiments on guinea pigs indicate that this species is also susceptible to inoculation with pox virus.

The first experiment comprised 6 animals which were injected intratesticularly, intraperitoneally, or intranasally with 0.1 to 0.4 cc. doses of testicular tissue filtrate derived from the 2nd rabbit passage of the Xy171 strain of virus (1). In the 2 guinea pigs injected intratesticularly there was a moderate enlargement and resistance of the testicles which developed on the 2nd day, persisted for 3 or 4 days, and then subsided. The other animals showed no clinical effect of the injections. Post mortem examinations on the 9th and 15th days were negative except in the case of the liver of 1 guinea pig killed on the 9th day. The organ appeared normal except that on the surface there were several small greyish opaque

spots about a millimeter in diameter. The significance of these areas is not entirely certain, but they may have been pox lesions. The subinoculation of rabbits with this liver material gave inconclusive results.

In the second experiment, 2 guinea pigs were injected intradermally with an 0.3 cc. dose of a Berkefeld V virus filtrate derived from testicular tissue of a rabbit in the 5th rabbit passage of the Xy171 strain. The clinical and post mortem results on these animals were likewise negative.

For the third experiment an unfiltered virus emulsion prepared from the testicles of a rabbit of the 10th serial passage of the Xy171 strain of virus was used (1). 2 guinea pigs were injected intratesticularly with 0.5 cc. and 2 intraperitoneally with 1.0 cc. In addition, each animal was injected intradermally on a shaved area of the side of the body with a dose of 0.3 cc. A definite cutaneous reaction developed in each animal which was considered positive. There was some swelling and reddening of the skin on the day following injection. By the 5th day these areas measured 1.5 to 2.0 cm. in diameter, they were definitely congested and indurated with a small central crust. One of the lesions was also edematous and in this case the reaction increased in intensity during the next 2 days. Regression of all lesions was well under way 8 days after inoculation at which time the animals were killed.

The 2 guinea pigs injected intraperitoneally showed no general signs of infection and post mortem examination 8 days after inoculation was negative. In both intratesticularly injected animals swelling and induration of the testicles developed on the day after inoculation and persisted for 3 or 4 days. Post mortem examination on the 8th day was negative except for slight congestion and enlargement of the testicles. The testicles of one animal were ground with Locke's solution and alundum to make a 10 per cent emulsion. Cultures of the emulsion showed no growth. A rabbit injected intratesticularly with 1.0 cc. doses of the unfiltered emulsion developed a typical case of the fulminating type of experimental pox infection (1). The reaction was characterized by fever which on one occasion reached 106.3°F. and an acute marked hemorrhagic orchitis and scrotal edema. On the 8th day the animal was seriously ill and was killed.

In the fourth experiment 2 guinea pigs were injected intratesticularly with 1.0 and 0.5 cc. doses of an unfiltered emulsion prepared from the testicles of a rabbit of the 15th consecutive filtrate series of the Xy171 strain of virus. The emulsion was bacteriologically sterile. Both animals developed swelling and induration of the testicles and in one case there was slight scrotal edema. 1 guinea pig was killed on the 8th day; the testicles were congested and the parenchyma swollen. The 2nd animal was found dead on the 4th day; the testicles were swollen, indurated, and hemorrhagic. An emulsion of the left testicle from the latter animal was prepared in the usual manner; bacteriologic examinations were negative. The following subinoculations of unfiltered emulsion were made. A rabbit was injected intratesticularly with 1.0 cc. doses, 2 guinea pigs were injected intratesticularly with 0.5 and 1.0 cc. doses respectively, and a 3rd guinea pig was injected intradermally with 0.2 cc. In the case of the rabbit, the results were typical of the fulminating type of experimental pox infection. A hemorrhagic orchitis and

scrotal edema developed rapidly and reached massive proportions on the 4th day, fever was observed on the 3rd, 4th, and 5th days, on the latter day a generalized cutaneous papular rash developed, and on the 7th day there was a bilateral blepharitis and conjunctivitis, a profuse watery nasal secretion, and diarrhea. The animal was found dead on the 8th day.

Both guinea pigs injected intratesticularly with guinea pig tissue virus developed swelling and marked induration of the testicles 2 days after inoculation. The condition continued for 4 days and then began to subside. The animals were killed on the 12th day; post mortem examinations were negative except for some congestion of the testicular parenchyma.

The cutaneous reaction of the guinea pig injected intradermally comprised a swollen congested area 1.5 mm. in diameter on the day after inoculation. On the 2nd day the area was considerably larger and more congested, edema had developed, and the central portion was necrotic. On the 4th and 5th days the entire area was quite indurated and the necrotic portion was covered with a thin scab. Resolution began on the 6th day. On the 12th day when the animal was killed, healing was almost complete and only a small crusted thickening of the skin remained.

These results, although few in number, show that it is possible to recover pox virus from the testicles of guinea pigs injected intratesticularly 4 and 8 days previously with unfiltered rabbit tissue-virus. In addition, a 2nd generation of virus in guinea pigs was obtained by the use of testicular tissue obtained from a 1st generation animal and the intratesticular route of injection. In the case of both the 1st and 2nd generations, the presence of active virus was demonstrated by rabbit inoculation, the animals developing typical examples of clinical pox. The findings indicate that the continued serial passage of virus in this animal species is probably possible. While no generalized clinical manifestations developed in inoculated guinea pigs comparable to those of the rabbit (2), the death of one animal 4 days after inoculation of guinea pig tissue-virus suggests that the serial intratesticular passage of virus might be associated with a fatal outcome, as is the case in the rabbit (1).

The Calf Experiment

The susceptibility of the calf to rabbit pox virus was tested on one animal by the scarified skin route of inoculation. In this experiment which also included a number of rabbits similarly inoculated, the cutaneous reaction to pox virus was compared with the cutaneous reactions to two specimens of dermo- and one of neurovaccine. The

results of this comparison have already been reported in the third paper of this series in which other experiments with these four viruses, and in particular crossed inoculation and exposure experiments, are taken up (3). In the present instance the cutaneous reaction of the calf to pox virus will be discussed.

The calf was a Jersey Holstein 3½ months of age and 75 kilos in weight. A large area of skin on the right side of the body was shaved and scarified and 1.0 cc. of a 10 per cent unfiltered testicular tissue pox virus emulsion was rubbed on it. The testicular tissue was derived from the 17th consecutive passage of the Xy171 strain, the first 15 generations of which had been made with Berkefeld V filtered inocula (1). Similarly prepared areas on the left side of the body were each inoculated with a similar dosage of unfiltered vaccine virus emulsions, that is, of culture dermovaccine, of the New York City Board of Health vaccine, and of neurovaccine respectively.

For the first 2 days after inoculation, little change was noted in the pox inoculated area. The lines of scarification were at first a faint pinkish color which quickly faded so that the lines were barely discernible. On the 3rd day several small pinkish papules confined to the scarified lines were observed (Fig. 1); some of them had a small semitranslucent center indicative of beginning vesicle formation. On the 4th day there were several fresh papules. On the 5th day the number of papular lesions had greatly increased and there were a few which apparently did not develop in the scarified lines (Fig. 2). Several lesions were vesicular and the overlying skin showed beginning scaling. The older papules were much larger and many of them had necrotic hemorrhagic centers. In some of them the base and surrounding tissue were edematous.

During the 6th, 7th, and 8th days, all the lesions continued to become larger, and necrosis and hemorrhage were very marked. The majority were quite uniform in size. By the 8th day umbilication was especially prominent and practically all the lesions had tenacious blackish red crusts (Fig. 3). Edema was no longer present. Most of the lesions were discrete but a few had coalesced. On the 10th day the lesions felt firmer and dryer, there was no apparent increase in size, and regression was thought to have begun (Fig. 4). The crusts had become very thick and tenacious. A few lesions, and these were the ones which had formerly been vesicular with little or no hemorrhage and necrosis, felt fibrous and dry and continued to show a fine dry scaling. During the following 5 days regression of the lesions continued as shown by a definite decrease in size, but they were still large and prominent with attached thick crusts on the 15th day when the animal was killed. Post mortem examination revealed no gross abnormalities of the viscera.

The rectal temperature was taken twice daily. Values of 102.0-102.6°F. were observed on the 6th to the 9th days inclusive, in contrast to the readings of 100.8-101.8°F. (mean value 101.3°F.) recorded prior to and after this period. On the 7th, 8th, and 9th days the animal appeared listless, there was some loss of

appetite, and a rather profuse and frequent watery diarrhea developed. Definite improvement was noted on the 10th day and by the 15th day the general condition seemed excellent. During the fortnight of the experiment there was a gain in weight of 2.2 kilos. It should be remembered, however, that the significance of these general symptoms as an indication of a positive reaction to inoculation of pox virus is complicated by the fact that the reactions to 2 strains of dermovaccine and 1 of neurovaccine inoculated on the opposite side of the body were also positive, as has already been described (3). It is of interest to note, however, that the lesions induced by these three vaccine viruses were in each case very much less pronounced and persistent than those induced by pox virus.

The results show that under the conditions of this experiment the calf is susceptible to inoculation with rabbit pox virus by the scarified skin route as far as the development of papules and vesicles in the inoculated area is concerned. The few vesicular lesions were comparatively small and typical pustule formation did not develop. The majority of lesions were large papules in which necrosis and hemorrhage and to some extent edema were very prominent features. The lesions appeared to be active up to the 10th day; they were still conspicuous although definitely regressing on the 15th day when the experiment was terminated. It should be pointed out that necrosis, hemorrhage, and edema were characteristic and conspicuous features of the reaction of the rabbit to inoculation with pox virus. They were regularly seen in the testicle after intratesticular injection of virus and in the cutaneous lesions induced by intradermal injection; they were also observed in the scarified skin inoculated with unfiltered virus emulsions, and in addition frequently occurred in the generalized papular eruption of the skin (2).

GENERAL DISCUSSION

The experiments reported in this and previous papers (1–4) were initiated by an epidemic disease of great severity which broke out in a rabbit breeding colony in December, 1932, (5). The pock-like character of the generalized cutaneous eruption was a conspicuous feature and suggested the name "rabbit pox." In the circumstances of this clinical manifestation the idea that vaccine virus might be the etiological agent was naturally considered although many features of the disease, and in particular the severe prostration and high mortality, were not those generally associated with vaccinia, especially under

conditions of spontaneous infection. In addition, no source of vaccine virus was evident at the time. There have been epidemics in rabbits, however, which have been attributed to vaccinia and particularly neurovaccinia, but satisfactory descriptions of the conditions and precise identification of the etiological agents are not available.

The experimental investigations carried out in connection with the pox epidemic were chiefly concerned with the isolation and identification of the causative agent.

A filterable agent was isolated from spontaneous cases and passed serially in rabbits without appreciable change in its pathogenic properties (1). The virulence of the virus, furthermore, was retained under conditions of ice box storage of affected tissues with or without the addition of glycerol. The clinical picture of the disease induced in normal rabbits by inoculation of tissue-virus was indistinguishable from that seen in spontaneous pox (2).

Rabbits which had recovered from the spontaneous or the experimental disease were refractory to inoculation of pox virus, nor did clinical manifestations of the infection develop under conditions of exposure to florid cases (3). Furthermore, the serum of pox recovered rabbits completely neutralized pox virus (4). The results of crossed inoculation and immune serum-virus neutralization experiments showed that there was no specific relationship between the viruses of pox and virus III disease (3, 4). A similar conclusion was drawn from the experiments on pox and infectious myxoma of rabbits (3). In the case of vaccinia, however, some relationship between pox and vaccine virus was evident although complete identity could not be demonstrated (3, 4). The relationship appeared to be closer between pox virus and neurovaccine than between pox virus and dermovaccine. It appeared probable that vaccination of rabbits with dermo- (culture) vaccine would be a satisfactory method of protection against rabbit pox.

Finally, as reported in the present paper, it was found that other animal species, namely, the mouse, the guinea pig, the rat, and the calf were susceptible to inoculation with pox virus. In mice, a fatal outcome within 5 to 6 days was regularly observed in the serial passage of virus by the method of brain to brain inoculation. In the calf, inoculation of scarified skin was followed by the development of large cutaneous papules with marked hemorrhage and necrosis. The reaction was much more pronounced than those observed with two specimens of dermovaccine and one of neurovaccine.

The epidemic of rabbit pox in the breeding colony did not spread to other animal species so far as is known and experiments were not carried out to determine whether other species could be infected with pox under conditions of room or cage exposure. It should be noted, however, that generalized clinical manifestations were not observed

in inoculated mice, rats, guinea pigs, or the calf, and consequently the possibility that these species might serve as carriers of the virus under natural conditions should be kept in mind.

In connection with the fatal infection in mice induced by intracerebral injection of rabbit tissue pox virus and subsequently seen in this species as a result of mouse brain to brain passage, the reaction of mice to intracerebral injection of vaccine virus is of particular interest.

Rosenau and Andervont (6) confirmed Levaditi and Nicolau's (7) failure to establish Levaditi's neurovaccine within the central nervous system of mice, but they succeeded with Armstrong's (8) modified dermovaccine carried in rabbits, a virus whose virulence had been greatly enhanced by a process of continuous heat selection and propagation (7). Mice injected intracerebrally with this virus died usually on the 6th or 7th day and serial transmission in mice was carried out by means of brain to brain transfers for 28 passages. Haagen (9) has recently reported experiments in which mice were regularly killed in 5 to 9 days by the intracerebral injection of a neurovaccine, the virulence of which for mice was fixed by continued mouse brain passages. This work was carried out with the highly virulent *Reichsgesundheitsamt, Berlin* virus, originally a rabbit strain of neurovaccine virus.

The results obtained from the intracerebral injection of mice with pox virus were of the same general order as those reported by Rosenau and Andervont and Haagen with 2 strains of vaccine virus, that is, an artificially modified dermovaccine and a neurovaccine, both of which are stated to be highly virulent. Whether the property of virulence alone is responsible for the observed results in mice is not entirely clear although Haagen's virus dilution experiments suggest that it plays a major rôle. In any event, it is evident that the present results on the susceptibility of mice to intracerebral injection of pox virus do not differentiate pox virus from certain vaccine viruses.

Is rabbit pox virus a variant or a mutant of dermo- or neurovaccine, or is it an independent member of the vaccinia group of viruses? In this connection it should be pointed out that the characteristic features of pox virus persisted in unabated form during the 9 months from January to October, 1933, in which the investigations on the virus and the experimentally induced disease were carried out. That modifications of dermovaccine may occur has been shown by Armstrong (8). The greatly enhanced virulence of this vaccine which was brought about by artificial means was found to persist in successive rabbit

passages. In the case of neurovaccine and dermovaccine viruses, there seems to be no doubt that in general behavior they are at least qualitatively different and that neurovaccine is the more potent or virulent. The potency of pox virus, however, was greater than that of neurovaccine and its immunity more powerful or effective. The results of the experiments reported in this series of papers clearly showed a relationship but not complete identity of pox virus with both dermo- and neurovaccine viruses; the relationship was closer in the case of neurovaccine. In this connection the possibility that neurovaccine virus is in reality rabbit pox virus or a derivative of it should be considered. Whether pox virus as it appeared in the colony was directly derived from a passage strain of dermovaccine or neurovaccine, or whether it was originally an independent member or strain of the vaccine group of viruses cannot be stated with finality. It was thought, however, that the characteristics of pox virus were such as to merit, at least for the present, an appropriate separate designation in the general group of vaccine viruses.

The manifestations of the spontaneous pox infection and of the experimentally induced condition were sufficiently individual to warrant the tentative consideration of a disease entity. The condition differed in many respects, and markedly so in severity, from vaccinia as generally reported and from vaccinia as recently observed in this laboratory (10). With respect to neurovaccinia, the differentiation is not as definite. From the available literature, comparative estimates of the character and severity of spontaneous neurovaccinia infections are difficult to arrive at and our own experience with induced neurovaccinia is much less extensive than with pox. But from the observations which have been made, rabbit pox as it occurred in 1932–33 was a much more severe disease. Taking into consideration the spontaneous infection and its epidemic features, together with the characteristics of the etiological agent and of the experimentally induced infection, should this disease be considered a pox disease of the rabbit in the sense of vaccinia of the calf, grease of the horse, pox disease of sheep, and small pox of man? A search of the medical and veterinary literature has failed to reveal any reference to rabbit pox as such. On the continent of Europe, however, a disease of the rabbit is recognized which is called *Pocken* and which greatly resembles

or is identical with pox as it appeared in our colony.[1] The present evidence is clearly not sufficient to speak with certainty, but the fact that there are two such similar, if not identical, diseases of apparently independent existence, one in Europe and the other in America, supports the idea of rabbit pox as a disease entity.

SUMMARY

The white mouse, the guinea pig, the calf, and probably the rat, were found to be susceptible to infection with the virus of rabbit pox.

Serial transmission of the virus in mice by brain to brain passage was characterized by a fatal outcome usually on the 5th or 6th day after inoculation.

Infection of the guinea pig was accomplished by intratesticular injection and the virus was continued to the 2nd passage in this species. Guinea pigs developed a well marked cutaneous reaction from the intradermal injection of both rabbit and guinea pig tissue virus.

Active virus was demonstrated in the testicles of rats 8 days after intratesticular injection by rabbit subinoculation.

In the calf inoculation of the scarified skin was followed by the development of large papular lesions with marked hemorrhage and necrosis.

The results of the investigations on the etiology of rabbit pox and of the experimentally induced infection reported in this and the four preceding papers (1–4) are discussed with special reference to the relation of pox virus to other viruses and of rabbit pox to other pock diseases.

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[1] Mahlich's (11) handbook for rabbit breeders and fanciers contains the following description (translation) of this condition:

"*Pox.*—The disease fortunately occurs very rarely in our rabbit population. It is characterized by a rash which involves the entire body. The papules are about the size of a hemp seed and at first are filled with a water-like content. Within a few days this changes into pus. The pocks which are now mature rupture and there remains a brownish base.

"An efficient treatment for this disease is difficult to attain. The best remedy which we know for pox is and remains the following: 'Make the sick rabbit shorter by a head.'"

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EXPLANATION OF PLATE 37

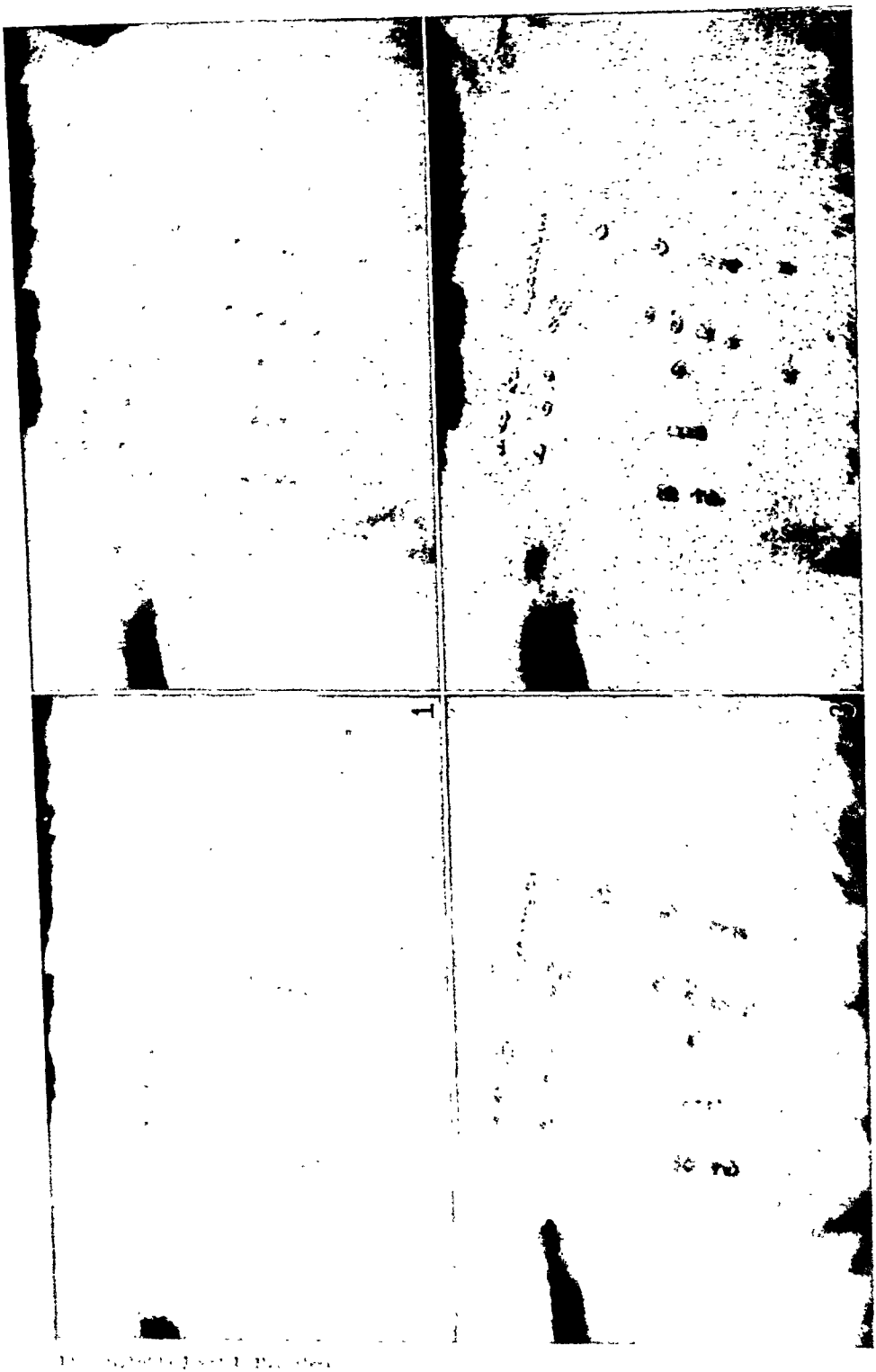
The cutaneous reaction of the calf to inoculation with rabbit pox virus. 1.0 cc. unfiltered tissue virus emulsion was rubbed into a scarified skin area on the side of the body.

FIG. 1. 3 days. Several small pinkish papules in the lines of scarification. Some of the lesions show slight vesicle formation.

FIG. 2. 5 days. The papules have increased in size and many new lesions have developed. A few are not definitely in the scarified lines. Hemorrhage and necrosis are prominent features of many lesions and edema is also present in some of them. There are a number of small umbilicated vesicles containing a semi-translucent whitish material; the overlying skin is beginning to scale.

FIG. 3. 8 days. Large umbilicated papules with pronounced necrosis and hemorrhage and crust formation. While most of the lesions are discrete, some have coalesced.

FIG. 4. 10 days. Beginning regression. The lesions are firmer and dryer with thick tenacious black or reddish black crusts. Some of them show fine scaling of the overlying skin.



STUDIES ON AN UNCOMPLICATED CORYZA OF THE DOMESTIC FOWL

V. A Coryza of Slow Onset

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The type of fowl coryza with which the bacterium *Hemophilus gallinarum* is associated has, in our experience, been characterized by a uniformly short incubation period. In normal birds which are confined indoors the interval between the injection of either exudate or the specific bacillus in pure culture and the appearance of a nasal discharge has rarely been longer than 2 days. In sharp contrast to the rapid onset of this type of coryza is the delayed incubation period of another infectious coryza which has been encountered from time to time in birds injected with nasal exudate from naturally infected fowl. Earlier observations on this coryza, in 1932, had suggested that its characteristics were not stable since the continued passage of exudate in normal fowl had been followed by reversion to the coryza of rapid onset.[1] Additional strains of the second type were not immediately available and further investigation was not begun until the fall of 1933 when an attempt was made to determine whether or not the coryza of slow onset was a distinct entity or a variant of the coryza of rapid onset.

Serial Passage of the Coryzas of Rapid and Slow Onset

Recently isolated strains of the coryzas of rapid and slow onset were carried simultaneously through a long series of passages in normal fowl as a test of the stability of their respective characteristics. A rigid quarantine of the infected birds was observed as a precaution against accidental cross infection or the introduction of infection from outside

[1] Nelson, J. B., *J. Exp. Med.*, 1933, 58, 297.

sources. The experimental birds were drawn from a flock of Rhode Island Red fowl reared and maintained at The Rockefeller Institute in Princeton. No natural cases of upper respiratory tract disease have ever been noted in this stock which has been under observation for many generations.

In November, 1933, exudate removed from the nasal passages of several naturally infected birds from a nearby poultry farm was injected into normal fowl and found to produce a coryza of slow onset. The disease was subsequently maintained by serial passage, at intervals which varied from 2 to 5 weeks after the appearance of a nasal discharge. Exudate obtained from the anterior nares

TABLE I

The Incubation Period of the Coryza of Rapid Onset

No. of birds	No. of passages	Date of first passage	Date of last passage	Incubation period		
				24 hrs.	48 hrs.	72 hrs.
54	40	Sept. 13, 1933	July 10, 1935	35	18	1

TABLE II

The Incubation Period of the Coryza of Slow Onset

No. of birds	No. of passages	Date of first passage	Date of last passage	Incubation period in days				
				9–11	12–14	15–17	18–20	21–31
72	20	Nov. 18, 1933	July 9, 1935	8	31	14	6	13

during life or from the interior nasal passages at autopsy was well mixed with sterile nutrient bouillon and approximately 0.5 cc. introduced into the palatine cleft of one or more susceptible fowl. A capillary pipette with an attached rubber hose was used in making the injections.

A strain of the coryza of rapid onset obtained in September of the same year from another local farm was maintained in much the same way. During the first year of observation exudate from the birds in this group was being used for other experiments. For this reason the number of passages was greater than with the coryza of slow onset although fewer birds were actually employed.

Following the injection of exudate the birds were observed daily until a nasal discharge appeared. As already noted, the infected birds which were usually 2–3 months old when injected were held under strict quarantine in separate units to which only the writer and his assistant had access. The birds were generally housed in individual cages, but in some cases 2 birds were kept in a single cage.

Data on the duration of the respective incubation periods of the two types of coryza are summarized in Tables I and II. 40 passages

of the coryza of rapid onset were made between September, 1933, and July, 1935. 54 birds are included in this series of passages. The interval between the injection of exudate and the appearance of a nasal discharge was 1 day in 35 cases (65 per cent), 2 days in 18 (33 per cent), and 3 days in a single case (1.0 per cent). 20 passages of the coryza of slow onset were made in 72 birds between November, 1933, and July, 1935. The incubation periods were 9 to 11 days in 8 cases (11 per cent), 12 to 14 days in 31 (43 per cent), 15 to 17 days in 14 (19 per cent), 18 to 20 days in 6 (8 per cent), and over 20 days, up to 31 as a maximum, in 13 (18 per cent).

Duration of the Coryzas of Rapid and Slow Onset

Birds infected with local strains of the coryza of rapid onset have generally shown a nasal discharge for 2 months and often longer. Infection with one strain, however, caused a coryza which in 20 fowl averaged only 11 days in duration (1). It should be noted that infection with these strains was established by the injection of exudate.

The duration of the nasal discharge in birds infected with the earlier strains of the coryza of slow onset was also prolonged. Observations on the birds infected with the present strain are in accord with this finding. The majority of the infected birds were brought to autopsy early in the disease. 12, however, were kept under observation for a month after the appearance of a discharge and 8 until the discharge had subsided. Of the former, all showed a continuous discharge until killed. Of the latter, the duration of the coryza was 8 weeks in 3 cases, 10 to 12 weeks in 3 cases, and 20 weeks in 2 cases.

Prolonged Manifestations of the Coryzas of Rapid and Slow Onset

The pathological features of the two coryzas are nearly identical. The only observed difference between them concerns the nature of the exudate which is poured forth in the nasal passages. This difference is not a constant finding, however, and has little diagnostic value.

In both cases the chief symptom of the disease is a nasal discharge and the characteristic postmortem finding the presence of exudate in the nasal passages and somewhat less regularly in the adjoining orbital sinuses. Early in the disease the exudate which partially fills the nasal passages of birds infected with the coryza of rapid onset is consistently mucopurulent. Microscopically it shows numerous polynuclear leucocytes with relatively few epithelial and mononuclear cells. The

exudate which the coryza of slow onset calls forth may also be mucopurulent but not uncommonly it is more catarrhal in nature: of a definitely heavier consistency, less readily drawn up with a pipette, and richer in cells—which are predominantly tissue cells. Polynuclear leucocytes may always be found but are usually scarce. Both types of coryza may exceptionally be accompanied by conjunctivitis and tracheitis. The rarity of these manifestations, it may be noted, has characterized all of the strains of both types which have been obtained locally.

Bacteriological Findings with the Coryzas of Rapid and Slow Onset

Cultures were made at irregular intervals from the nasal exudate of birds infected with the coryza of rapid onset. The nasal exudate was streaked on the surface of a horse blood agar plate which was sealed prior to incubation. Colonies of the fowl coryza bacillus, *Hemophilus gallinarum*, were always obtained. If the exudate was cultured during the first few days after the appearance of a nasal discharge, a nearly pure growth of the specific bacillus was generally secured. Cultures made late in the disease showed, in addition, numerous colonies of miscellaneous bacteria. The cultures obtained from these birds were in no way different from the earlier described strains of the fowl coryza bacillus. Like the earlier strains, they all failed to colonize on unsealed blood agar plates and injected intranasally in susceptible fowl they all produced a coryza of short duration.

Cultures were also made from the nasal tract of 50 birds infected with the coryza of slow onset, using both open and sealed blood agar plates. In most cases the exudate was removed for cultivation during the first week after the appearance of a nasal discharge. The plates generally showed miscellaneous colonies, in varying numbers, characteristic of the normal bacterial flora of the upper air passages. Occasionally no growth was obtained on either plate. The conspicuous finding, however, was the complete absence of colonies of the fowl coryza bacillus on the sealed plates. Although most of the cultures were made at a particularly favorable time for the detection of that organism it was not obtained in a single instance.

DISCUSSION

The distinguishing features of the coryza of slow onset appear to be sufficiently stable and distinctive to justify separating it from the coryza of rapid onset. The incubation periods of the two coryzas, although subject to some variation, have never overlapped during the

period that they have been under observation. The interval preceding the appearance of a nasal discharge has not exceeded 3 days in birds infected with the coryza of rapid onset and has not been less than 9 days in birds infected with the coryza of slow onset. There is, of course, no reason to believe that the actual time limits which have obtained with this particular stock of birds would be duplicated in some other genetically different stock.

Substantial support for the separation of the two coryzas is afforded by the bacteriological findings with respect to the fowl coryza bacillus. This organism was readily and regularly isolated from the nasal exudate of birds infected with the coryza of rapid onset but was never obtained from birds infected with the coryza of slow onset. In the case of the earlier studied strains of the latter type attempts to isolate the fowl coryza bacillus had also failed as long as the infected birds showed a long incubation period. These earlier strains, however, finally changed to a coryza of rapid onset and this change was accompanied by the appearance of *Hemophilus gallinarum* in the nasal discharge. In the light of the present observations, it seems probable that this change was accidental and not a true reversion. The change may have been referable to a latent infection with the specific bacillus or more probably to the chance carriage of infection from one group of birds to the other. It can be said with certainty, however, that it was not due to the presence of bacterial carriers in the normal stock of birds.

SUMMARY

A strain of fowl coryza of slow onset was carried through 20 successive passages in susceptible birds over a period of approximately 19 months. During this period it retained its initial characteristics as did also a coryza of rapid onset which was similarly maintained. 88 per cent of 72 birds infected with the coryza of slow onset showed a nasal discharge after an incubation period of 12 days or more; the actual limits being 9 to 31 days. 98 per cent of 54 birds infected with the coryza of rapid onset showed a nasal discharge on the 1st or 2nd day after injection. The duration of both coryzas was prolonged.

Bacteriological examination indicated that *Hemophilus gallinarum* which is invariably present in the nasal exudate of birds infected with the coryza of rapid onset is not associated with the coryza of slow onset.

STUDIES ON AN UNCOMPLICATED CORYZA OF THE DOMESTIC FOWL

VI. Coccobacilliform Bodies in Birds Infected with the Coryza of Slow Onset

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PLATE 38

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In the preceding paper evidence was presented that the coryza of slow onset is a stable entity which is apparently unaffected by long continued serial passage in susceptible fowl (1). It cannot be established as an independent disease, however, until its etiology is elucidated. The only pertinent information concerned the bacterium *Hemophilus gallinarum* which, it has been shown, is not present in the nasal exudate of infected fowl (1). Maintenance of the coryza of slow onset was, accordingly, continued by serial passage and an effort made to determine its causative agent.

The Bacteriological Examination of Exudate

The cultivation of exudate from birds infected with the coryza of slow onset on blood agar plates revealed no bacteria which had not been previously isolated. The organisms which were encountered colonized freely on open as well as on sealed plates and also grew vigorously in fluid media enriched with blood. The bacteria which were most frequently met with fell into three groups, namely: diphtheroids, Gram-positive cocci, and Gram bacilli. There was no uniformity in their appearance from bird to bird and the pure growth of a single species was rarely observed. They were usually few in number during the first week or two after the onset of symptoms, increased as the coryza progressed, and became very numerous late in the disease. In some cases, however, exudate from the orbital sinuses, which

subsequently proved to be infective, failed to show colony formation on either sealed or open blood agar plates. As the result of past experience with these bacteria it can be stated that all of them are secondary invaders and of no direct etiological significance.

In the routine performance of autopsies on infected birds a presumptive appraisal of the bacterial flora of exudate was made by direct films which were Gram stained. If the films showed no or few bacteria the exudate in addition to being plated was also cultured in fluid horse blood at the base of slanted nutrient agar. In some cases the plates showed no growth, nor did the solid portion of the slant culture but the fluid portion of the latter contained a minute Gram organism which will subsequently be referred to as the X bacillus.

The X bacillus is an extremely small organism, 0.5μ or less in diameter. It is tentatively designated a bacillus but it might equally well be called a coccus for the cells are predominantly spherical and only occasionally elongated. It is Gram-negative and non-motile. The cells are almost invariably grouped in clumps which may become large and compact comprising innumerable units. Growth in a fluid nutrient medium containing blood is slow and scant. Characteristic clumps are usually present on the 2nd day and regularly on the 3rd day, after incubation at 37°C. The clumps are never numerous and their detection may require considerable searching. After the 3rd day degenerative changes, which may be accompanied by swelling, set in and the cells begin to lose their sharpness of outline. Recently isolated cultures have failed to colonize on open or sealed blood agar plates. After repeated transfers, however, pin point colonies may be produced on the surface of both plates. One strain of the bacillus which has been carried through 80 biweekly or weekly subcultures still fails to colonize.

For a brief period it was believed that the X bacillus was of some etiological significance since it was found that the initial culture of one strain was infective for normal birds. This view was soon shown to be erroneous. Subcultures of this strain were innocuous as were the primary cultures of other strains. The infectivity of the single culture was probably due to the presence of exudate in the material which was injected as it was later observed that primary cultures containing exudate which was culturally sterile would occasionally infect normal birds. Isolation of the X bacillus was, moreover, irregular; in all only 8 cultures were obtained, an incidence of roughly 10 per cent.

The Filtration of Exudate

Since there was no indication that a cultivable bacterium was directly concerned in the etiology of the coryza of slow onset, attention was directed to the filterable viruses. Exudate from affected birds was, accordingly, filtered through Berkefeld V candles and the filtrate tested for infectivity.

The exudate was removed from the interior nasal passages of 2 or more infected fowl, thoroughly mixed with approximately 10 cc. of nutrient bouillon, and filtered through Berkefeld V candles (3.5 cm.). 3 separate filtrations were made. In 2 instances the suspensions were passed through candles known to be impermeable to *Hemophilus gallinarum*. In one instance the exudate mixture was divided into two portions; one of which was filtered through a similarly impermeable candle and one through a candle which failed to hold back the test organism. All of the filtrates were tested for sterility by adding a 0.5 cc. portion to an equal volume of defibrinated horse blood at the base of a nutrient agar slant. None of the filtrate cultures showed either macroscopic or microscopic evidence of growth. The exudate was cultured on blood agar plates prior to filtration and in each case showed numerous colonies of miscellaneous bacteria. The filtrates were tested for infectivity by the intranasal injection of 0.5 cc. portions in normal fowl, which were 2–3 months of age. A similar portion of unfiltered exudate was also tested in each case. Unless the injected bird showed a discharge at an earlier time it was kept under observation for at least 4 weeks. It was then autopsied and the nasal tract examined.

The results of the filtration experiments are summarized in Table I. The three groups of birds which were injected with exudate filtered through candles impermeable to *Hemophilus gallinarum* all remained normal during the period of observation and at autopsy, 33 to 42 days after injection, showed no indication of a nasal involvement. The 3 birds which received unfiltered exudate showed a nasal discharge after 12 to 14 days. One of the 2 birds which were injected with exudate filtered through a candle which was permeable to *Hemophilus gallinarum* showed a nasal discharge on the 27th day after injection. At autopsy, on the following day, a large amount of mucopurulent exudate was found in the nasal passages and a smaller amount in both orbital sinuses. 2 blood agar slant cultures made from the left sinus showed no growth macroscopically or microscopically. The 2nd bird failed to show a nasal discharge but at autopsy on the 33rd day a

quantity of mucopurulent exudate was found in the right orbital sinus. This exudate was also culturally sterile. Normal fowl which were injected with exudate from these 2 birds, Nos. 11 and 12, showed a nasal discharge on the 21st and 25th day, respectively.

The Microscopic Examination of Exudate

In the microscopic examination of films made directly from exudate it had been noted that minute coccobacilliform bodies were often

TABLE I

The Infectivity of Filtered Exudate

No. of exudate	Type of filter	No. of bird	Material injected	Result of injection
1	Impermeable V	1	Filtered exudate	Normal 33 days
		2	" "	" 33 "
		3	Unfiltered exudate	Nasal discharge 14th day
2	" "	4	Filtered exudate	Normal 36 days
		5	" "	" 36 "
		6	Unfiltered exudate	Nasal discharge 12th day
3 A	" "	7	Filtered exudate	Normal 42 days
		8	" "	" 42 "
		9	" "	" 42 "
		10	Unfiltered exudate	Nasal discharge 12th day
3 B	Permeable V	11	Filtered exudate	" " 27th "
		12	" "	Sinusitis at autopsy on 33rd day

present, being particularly conspicuous in exudate which did not contain the usual secondary bacteria (2). These bodies were frequently arranged in aggregates which were morphologically indistinguishable from those characterizing the X bacillus in culture. It was soon apparent, however, that the coccobacilliform bodies which were present in films could not be identical with the bacillus obtained in cultures. As more attention was paid to the detection of these bodies it was found that they were almost invariably present in films, whereas the X bacillus was only rarely isolated in cultures.

The coccobacilliform bodies are minute immobile cells which may be found as single or double units and in aggregates varying from a few cells up to large compact masses of innumerable units. In shape they are commonly spherical but may be slightly elongated, appearing as extremely short bacilli. In size they are generally under 0.5μ and may be so minute that they are barely visible. In exudate removed early in the disease the bodies are usually quite uniform in size. Their detection in films requires a rather intense stain. For routine purposes the Gram stain, counterstaining for several minutes with carbolfuchsin diluted 1:4 with water, has proved highly satisfactory. The individual cells stain deeply and stand out sharply. The bodies are also well stained by Victoria blue. Giemsa stain has given less satisfactory results. The outline of the individual bodies tends to be fuzzy and they may be confused with precipitated material. The color with Giemsa is a purplish shade.

The most favorable time for the detection of the coccobacilliform bodies is during the first week after the appearance of a nasal discharge. Later in the disease they tend to be obscured by secondary bacteria which may be present in large numbers. The bodies are predominantly extracellular regardless of the stage of the disease. They may also be found intracellularly, chiefly in polynuclear leucocytes but occasionally in epithelial cells. They vary greatly in numbers even when films are made shortly after the onset of the nasal discharge. If the exudate is mucopurulent in nature, with numerous leucocytes, the bodies are commonly well distributed throughout the film, generally extracellular, and in small groupings. Occasionally large cloud-like masses of the bodies may be observed. In some cases, however, they are scarce in such films and their detection may require considerable searching. In the thick catarrhal type of exudate which contains many epithelial cells but few leucocytes they are always present in small numbers. In such exudate the bodies are sometimes found within epithelial cells but this is a rare occurrence. In the lacrimal ducts they may be present in considerable numbers in the absence of leucocytes. The detection of the bodies in older cases of coryza which show large numbers of miscellaneous bacteria in the nasal canals and in the orbital sinuses may often be made from the lacrimal duct films which usually contain relatively few secondary bacteria. Well defined bodies have been found in the lacrimal ducts as long as 2 months after the onset of symptoms.

Cells similar to the coccobacilliform bodies have not been found in

films from the nasal mucosa of normal fowl, or in the exudate from birds infected with cultures of *Hemophilus gallinarum*. They are found so regularly in the exudate from birds affected with the present strain of coryza that they have come to be regarded as characteristic of it as is the prolonged incubation period. In addition to their presence in birds infected by intranasal injection they have also been found regularly in birds which have acquired a coryza by direct contact. That they are not peculiar to this particular strain of the coryza of slow onset is indicated by their detection in birds affected with 2 other strains. These particular strains were obtained originally, in the spring of 1935, from naturally infected fowl and were for some time maintained by serial passage. Coccobacilliform bodies have also been found in birds infected with what was supposedly a pure strain of the coryza of rapid onset, but which in all probability was a mixture of the two types of coryza.

All attempts to grow the coccobacilliform bodies in artificial media have been unsuccessful. Infective exudate, known to be free from bacteria, has been cultivated under aerobic and anaerobic conditions at 37° and 42°C. using both solid and fluid media enriched with a variety of substances as defibrinated blood, serum, amniotic fluid, and yeast extracts. Save for the occasional isolation of the X bacillus no indication of growth has ever been noted. The bodies have, however, been cultivated in fertile eggs and in tissue cultures employing chick embryo tissue. The latter observations which are still incomplete will be considered in a separate report.

DISCUSSION

Etiological analysis of the coryza of slow onset has been retarded by the prolonged incubation period which has necessitated the observation of experimental birds for long periods of time. It was soon evident that the cultivable bacteria were of no direct causal significance. *Hemophilus gallinarum* was readily excluded since it was never isolated from nasal exudate. The different species of free growing bacteria which were isolated from the nasal tract could all be regarded as secondary invaders. The so called X bacillus was ruled out as an etiological factor by reason of the rarity of its isolation and the failure of subcultures to infect.

Filtration experiments indicated that the infective agent of the coryza was incapable of traversing the usual Berkefeld V filters but that it could pass through a V filter which was permeable to *Hemophilus gallinarum*. This observation suggested that the causative factor was either a virus of large size or a bacterium of small dimensions. The usual but not invariable association of polynuclear leucocytes with the coryza, even during the early days of the disease, tended to contraindicate a virus as the causal agent. Accordingly, attention was focused on certain minute coccobacilliform bodies which had been noted in films made directly from exudate.

The present report has dealt with the characteristics of these bodies, emphasizing their almost invariable detection in stained films, their extracellular predominance, and their apparent inability to grow in or on artificial media. The etiological significance of such an agent, which cannot be cultivated by the usual bacteriological methods, is difficult to establish. The present evidence concerning its relation to the coryza of slow onset is largely circumstantial but will be supported, in a later communication, by more concrete evidence gained by a study of the coccobacilliform bodies in fertile eggs and in tissue cultures.

The actual nature of the coccobacilliform bodies is undetermined. Morphologically they bear a resemblance to the elementary bodies of vaccinia and other virus diseases. It is of interest in this connection that Gibbs has recently reported the association of a filterable virus with an infectious fowl coryza (3). He found that the infective agent would pass through a collodion membrane with a pore size of 0.135μ but was held back by a membrane with a pore size of 0.082μ (4).

The coccobacilliform bodies are also morphologically suggestive of the rickettsiae. This group of infective agents possesses a number of characters in common with the elementary bodies. Resistance to physical and chemical agents, immunity relationships, antibody production, and particularly growth in an arthropod host are factors which serve to differentiate the known examples of these two groups but in general, however, they are not sharply separated. With an unknown infective agent it may be difficult to decide whether it should be classified with the filterable viruses, the rickettsiae, or neither. This situation which arose in connection with psittacosis, the causal factor

of which has been classified as a filterable virus (elementary body), a rickettsia, and a bacterium, is again encountered in the present instance. In the case of the coccobacilliform bodies it may prove to be of significance that their morphological features, which are distinctive, are similar to those of a cultivable bacterium isolated from the same source.

SUMMARY

Minute coccobacilliform bodies have regularly been found in the nasal exudate of fowl infected both by injection and by contact with the coryza of slow onset. These bodies are commonly less than 0.5μ in diameter and are predominantly extracellular. They have consistently failed to grow in artificial media. They are held back by Berkefeld V filters which are impermeable to *Hemophilus gallinarum* but may pass through filters which are permeable to the test organism.

The coccoid bodies are morphologically similar to a cultivable noninfective bacterium which may occasionally be isolated from exudate of the infected fowls.

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EXPLANATION OF PLATE 38

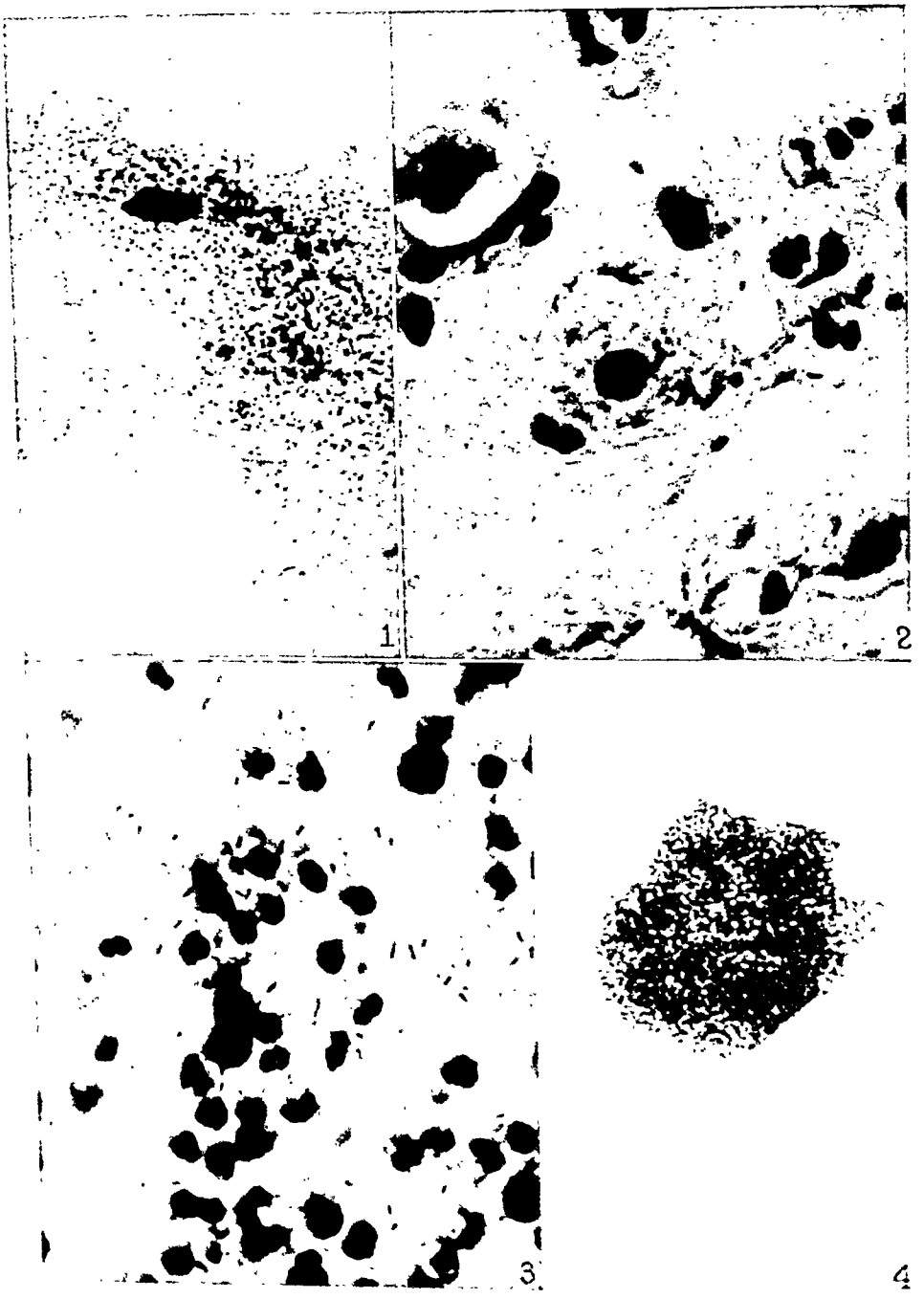
The preparations were all stained with Gram's method and magnified 920 diameters.

FIG. 1. Nasal exudate from a bird infected with the coryza of slow onset showing a cloud-like grouping of extracellular coccobacilliform bodies.

FIG. 2. Nasal exudate from a bird infected with the coryza of slow onset showing an epithelial cell containing several compact groupings of coccobacilliform bodies.

FIG. 3. Nasal exudate from a bird infected with a pure culture of *Hemophilus gallinarum* showing bacilli and leucocytes. The camera has reproduced a number of specks which were not conspicuous microscopically.

FIG. 4. Fluid from a 48 hour old blood agar slant culture of the X bacillus showing a compact clump of bacilli.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CONCERNING THE RELATION OF ENVIRONMENTAL TEMPERATURE TO RESISTANCE TO THYROID AND THYROXINE, AND THE CREATINE CONTENT OF THE HEART AND OTHER TISSUES IN EXPERIMENTAL HYPERTHYROIDISM

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Plates 39 and 40

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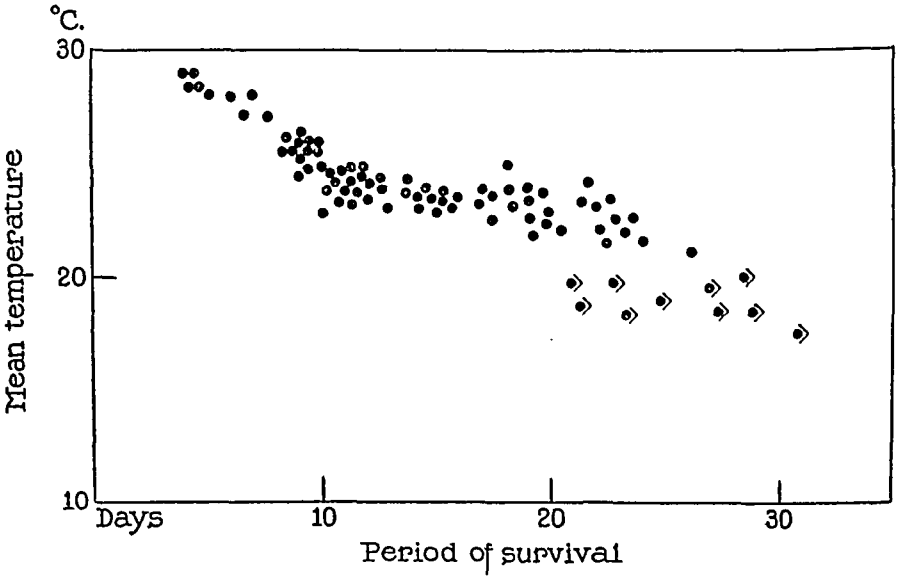
In 1919 Stoland and Kinney (1) published a brief statement concerning the relation of external temperature to the toxicity of administered thyroid. They found that rats kept at 32°C. and receiving 0.2 gm. of desiccated thyroid daily, lived an average of 7.3 days; others at 25°C. lived an average of 22 days, while a third group kept at 18°C. lived more than 32 days. A survey of the literature reveals no other reports specifically concerned with this question, although the more general problem of the relation of the environmental temperature to the structure and activity of the thyroid has been the subject of several investigations (2–9).

The present work is partly the outgrowth of the observation by one of us (10) that rats are much more resistant to thyroid and thyroxine in cool than in warm weather. It is improbable that this simple and obvious relationship has not been frequently observed by experimental workers, and certainly in clinical practice; yet it is remarkable that even in the more authoritative and comprehensive discussions of the physiology, pharmacology and therapeutics of the thyroid hormone, little or no mention is made of the importance of environmental temperature in relation to its tolerance and toxicity. For this reason the present report seems to be justified.

Methods

The rats used in this work were from a pure inbred Wistar strain reared in the laboratory under very favorable conditions. Special attention was given to the

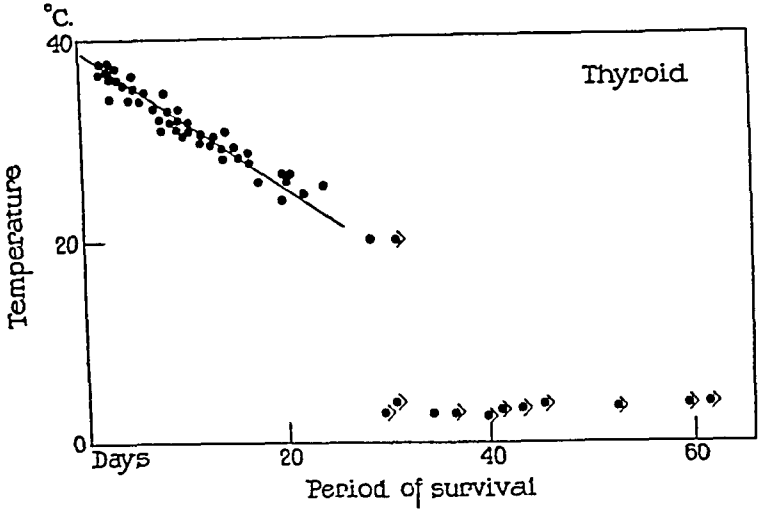
diet, particularly to the vitamin B requirement, as this is said to be greatly augmented in hyperthyroidism (11). One series of animals was observed at room temperature over a period of several months, during which time a daily record was kept of the extreme and mean temperatures. Another series was observed in a thermoregulated incubator at definite temperatures within the range of 20–37.5°C., while a third group was kept in a cold room at a temperature of 4–6°C. As the rat has naturally a high tolerance for thyroid, the daily dose of the desiccated preparation (U. S. P. Lilly) was usually fixed at 250 mg. per 100 gm. of body weight, although in some of the experiments at low temperatures the dose was increased to 1 gm. per rat per day. The thyroxine used was the crystalline, synthetic product



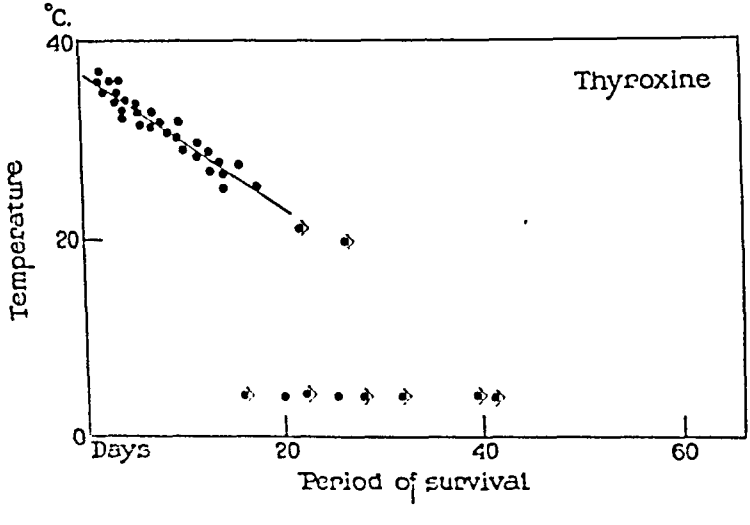
TEXT-FIG. 1. Showing the relation of mean environmental temperature to the survival period of hyperthyroid rats. Solid circles with arrows denote that the animals were alive at the conclusion of the experiments.

of Hoffmann-La Roche. For rats weighing approximately 200 gm., the daily dose was 2 mg., administered subcutaneously. However, essentially the same results have been obtained since with a daily dose of 1 mg. According to the recent observations of Lerman and Salter (12), 0.1 gm. of desiccated thyroid (Lilly, Armour or Lederle) produces approximately the same calorigenic effect in man as 0.3 mg. of pure thyroxine.

The weight of each rat was recorded either daily or every other day. At the termination of each experiment, the organs were removed, weighed and preserved for microscopic study. To add to the data previously reported (10, 13), and for reasons to be considered presently, estimations were made of the creatine content of the myocardium, liver, testes and the muscles of the hind limbs.



TEXT-FIG. 2



TEXT-FIG. 3

TEXT-FIGS. 2. and 3. Showing the relation of constant environmental temperature to the survival period of hyperthyroid rats. Solid circles with arrows denote that the animals were alive at the conclusion of the experiments.

RESULTS

The influence of external temperature on the resistance to thyroid and thyroxine is illustrated by the data charted in Text-fig. 1. As compared with a 4 to 5 day survival period when the mean room temperature was 28–29°C., rats kept at 20°C., or below, were alive at the end of 21 to 31 days. These observations therefore confirmed those of Stoland and Kinney (1). It was apparent, however, that the relationship represented in Text-fig. 1 was only roughly approximate, as there was often a difference of 2–3° between the daily mean and maximum temperatures, and even greater differences over a period of several days. For this reason Text-figs. 2 and 3, representing the data obtained at constant temperatures, give a closer approximation of the relationship of environment to the tolerance for the thyroid hormone.

It should be mentioned that both the incubator and cold room were unavoidably dark, but it is assumed on the basis of several investigations, notably those of Kenyon (9) and Mayerson (14), that the exclusion of light for moderate periods produces no demonstrable effects on the thyroid mechanism.

In the range of 20–37.5°C., the relation of temperature to the survival period was found to be almost linear. The tolerance was considerably increased at 20°C., but was especially striking at 4–6°C. Rats receiving large amounts of thyroid or thyroxine survived long periods of exposure in the refrigerator and even gained weight. One rat (290) was alive at the conclusion of the experiment, after remaining in the cold room for 99 days, during which time it consumed a total of 90 gm. of desiccated thyroid and gained 67 gm. in weight.[1]

The normal response to cold is increased metabolism, associated

[1] More recently observations have been made in a cold room maintained at 2–3°C. A proportion of the rats receiving thyroid or thyroxine, as well as their controls, succumbed within a relatively short period (15 to 30 days). In most of these animals loss of weight, edema of the extremities and pneumonia were prominent findings. Not a few chewed their tails and lost blood from hemorrhage. It is possible that the extreme cold, combined with the much higher moisture content of this cold room as compared with the one previously used, may account for the untoward results. As the data obtained in this refrigerator were inconstant, they have not been included in the present report.

with stimulation of thyroid activity. The characteristic changes in the gland are illustrated in Figs. 1 and 3.[2] The acini remain approximately normal in size, but there is an absence of colloid. The capillaries about them are widely distended. There is a piling up of the epithelium and some desquamation. The epithelium is high cuboidal to columnar. The nuclei are apparently normal in size, but stain very densely. These hyperplastic changes, together with the loss of colloid, are generally taken to mean a preponderant excretion from the thyroid gland and, according to Kenyon (9), may be diminished or prevented in rats exposed to cold by the daily administration of small amounts of iodide. In our work this was accomplished even more strikingly and uniformly by the administration of either desiccated thyroid or thyroxine. The glands of these animals showed enlarged acini filled with colloid which stained well (Figs. 2, 4, 5 and 6). The epithelium was flattened by the pressure of the excessive amount of colloid being stored. There was no apparent difference between the glands of animals fed thyroid and those receiving thyroxine.

A conspicuous effect of hyperthyroidism in the rat, induced by the administration of thyroid or thyroxine, is the marked reduction of the creatine content of the myocardium (10), frequently to about 50 per cent of normal. There is apparently a critical level below which the creatine concentration cannot be readily depressed. Thus far, in analyses of more than 200 hyperthyroid adult rats, the creatine concentration of the myocardium was found to be less than 70 mg. per cent only once, while in the majority of cases it was 90–100 mg. at the time of death. Under these circumstances collapse and death are usually sudden in occurrence, frequently following moderate exertion and often with the animal in a sitting posture, suggesting heart failure.

In view of the changes in the creatine content of the myocardium of rats treated with thyroid substance it was logical to inquire whether a similar change accompanied thyroid hyperfunction due to exposure to cold. However, analyses of the hearts of the eight control rats in this group (Table I) yielded values that were within the normal range. Work now in progress indicates that the creatine and creatinine excretion of rats exposed to a low thermal environment approaches levels

[2] The tissues were fixed in 10 per cent formalin, embedded in paraffin and stained with hematoxylin and phloxine.

attained by rats receiving thyroid or thyroxine. As the treated animals show marked depletion of the creatine reserves of skeletal and especially of cardiac muscle, it is to be concluded that the replacement of tissue creatine in this form of physiological hyperthyroidism is comparatively effective.

Although Cramer (5) has described a storing of colloid in the thyroid glands of rats and mice kept at higher temperatures than usual, his

TABLE I

Effect of Cold and of Thyroid and Thyroxine on Creatine Content of Rat Tissues

Rat No.	Died (D) or sacrificed (S)	Body weight		Duration of experiment	Creatine per 100 gm. tissue				Weight of ventricles	Creatine in ventricles	
		Initial	Final		Liver	Testes	Muscle	Heart (ventricles)			
		gm.	*gm.*	*days*	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*	*mg.*	
218 ♂	S	160	183	32	19	304	418	125	0.84	1.05	Total of 15 gm. of desiccated thyroid
219 ♂	S	164	165	32	21	354	435	105	0.80	0.84	Total of 60 mg. of thyroxine
220 ♂	S	160	221	32	21	327	454	184	0.75	1.38	Control
255 ♂	S	158	203	29	23	318	370	107	0.95	1.02	Total of 44 mg. of thyroxine
256 ♂	D	151	147	21	35	338	411	76	0.91	0.69	Total of 30 mg. of thyroxine
257 ♂	S	141	204	31	20	293	446	181	0.82	1.49	Control
288 ♂	S	188	238	53	26	—	485	120	1.02	1.22	Total of 45.5 gm. of desiccated thyroid
289 ♂	S	180	197	68	20	297	431	106	1.11	1.18	Total of 64.5 gm. of desiccated thyroid
290 ♂	S	191	258	99	22	329	452	121	1.22	1.47	Total of 90 gm. of desiccated thyroid
	S	223	211	27	20	308	483	177	0.83	1.47	Averages of 6 controls; 3 males and 3 females

experiments were of several hours duration only, or the animals were exposed to the higher thermal environment only a part of each day. In our work, the animals were kept at the higher temperatures continuously for several days. Under these conditions there was no evidence of storage of colloid either in the controls or in the rats treated with thyroid or thyroxine. As illustrated in Figs. 7 and 8, the glands

TABLE II

Effect of High Temperatures and of Thyroid and Thyroxine on Creatine Content of Rat Tissues

Rat No.	Temperature	Died (D) or sacrificed (S)	Body weight		Duration of experiment	Creatine per 100 gm. tissue				Weight of ventricles	Creatine in ventricles	
			Initial	Final		Liver	Testes	Muscle	Heart (ventricles)			
	°C.		gm.	gm.	days	mg.	mg.	mg.	mg.	gm.	mg.	
203 ♂	37.5	S	170	132	3	31	342	578	178	0.525	0.93	Control
204 ♂	37.5	D	170	127	3	48	383	545	126	0.509	0.64	Total of 1.2 gm. desiccated thyroid
211 ♂	37.5	S	168	135	4	30	305	490	206	0.421	0.87	Control
212 ♂	37.5	D	170	135	2	68	358	478	139.5	0.521	0.73	Total of 0.6 gm. of desiccated thyroid
213 ♂	37.5	D	172	140	2	74	308	486	151.5	0.512	0.78	Total of 2 mg. thyroxine
214 ♂	37.5	D	174	135	3	72	320	477	131	0.476	0.62	Total of 4 mg. thyroxine
215 ♀	37.5	S	155	144	4	27	—	491	197	0.463	0.91	Control
216 ♀	37.5	D	155	124	4	68	—	487	144.5	0.474	0.69	Total of 0.6 gm. desiccated thyroid
208 ♂	36	S	165	152	8	27	298	470	167	0.508	0.85	Control
209 ♂	36	D	165	130	2	82	375	491	138	0.520	0.72	Total of 1 gm. desiccated thyroid
210 ♂	36	D	175	136	3	50	310	464	113	0.550	0.62	Less than 1 gm. desiccated thyroid
224 ♂	34.5	D	163	134	3	33	327	528	122	0.466	0.57	Total of 3 mg. thyroxine
225 ♂	34.5	D	164	129	4	52	317	479	109	0.454	0.49	Total of 1 gm. desiccated thyroid
226 ♂	34.5	S	155	151	10	23	274	524	220	0.456	1.00	Control
332 ♂	33	D	229	176	4	37	366	537	121	0.704	0.85	Total of 8 mg. thyroxine
333 ♂	33	S	220	225	14	20	307	497	212	0.664	1.32	Control

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EXPLANATION OF PLATES

PLATE 39

FIG. 1. Thyroid of rat 220, control in the cold room, showing hyperplasia of epithelium and loss of colloid. Stained with hematoxylin and phloxine. ×240.

FIG. 2. Thyroid of rat 219, which received 60 mg. of thyroxine during 32 days in the cold room, showing storage of colloid. Stained with hematoxylin and phloxine. × 240.

FIG. 3. Thyroid of rat 257, control in the cold room, showing hyperplasia of gland and loss of colloid. Stained with hematoxylin and phloxine. × 240.

FIG. 4. Thyroid of rat 255, which received 44 mg. of thyroxine during 29 days in the cold room, showing storage of colloid. Stained with hematoxylin and phloxine. × 240.

PLATE 40

FIG. 5. Thyroid of rat 289, which received 64.5 gm. of desiccated thyroid during 68 days in the cold room, showing storage of colloid. Stained with hematoxylin and phloxine. × 240.

FIG. 6. Thyroid of rat 290, which received 90 gm. of desiccated thyroid during 99 days in the cold room, showing storage of colloid. Stained with hematoxylin and phloxine. × 240.

FIG. 7. Thyroid of rat 214, which received 4 mg. of thyroxine during 3 days at 37.5°C., showing edema and degeneration of the gland. Stained with hematoxylin and phloxine. × 240.

FIG. 8. Thyroid of rat 215, control in the incubator, showing edema and degeneration of the gland. Stained with hematoxylin and phloxine. × 240.

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AN EPIDEMIC IN A MOUSE COLONY DUE TO THE VIRUS OF ACUTE LYMPHOCYTIC CHORIOMENINGITIS

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PLATES 41 AND 42

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In a preliminary publication (1) the detection of a disease due to a filtrable virus in our colony of white mice was reported. The purpose of the present paper is to describe this disease in greater detail.

The Disease in Mice

Based on the number of immune mice, it was estimated that about 50 per cent of the mice of the colony were infected. The rates of morbidity and mortality cannot be given accurately; the former, however, was less than 20 per cent, and the latter less than 2 per cent of the number of infected mice. The majority of the infected stock mice showed no definite symptoms, and the infection was determined by inoculating their blood or emulsions of their brains into guinea pigs, or by testing the mice for immunity by intracerebral inoculation with virus, using equal numbers of mice from a virus-free stock as controls. Stock mice which proved resistant to intracerebral inoculation with virus very probably had acquired a true immunity following natural infection and were not merely naturally resistant to the virus, since mice of the same strain bred from virus-free parents are all susceptible to intracerebral inoculation with virus. That a subclinical, latent infection is the rule in mice became apparent from several experiments to be described in a following paper in which disease-free mice were infected by placing them in contact with infected ones.

In the infected colony it was noted that a number of 2 to 6 week old mice were emaciated and drowsy. Their fur was ruffled and they were often seen sitting in corners of the cage by themselves. Their movements were rather slow and stiff, and their legs appeared long in

proportion to their thin bodies. Nineteen mice presenting such symptoms (Group C, Table I) were examined for virus by inoculating suspensions of their brains or blood or both intracerebrally into guinea pigs, and the virus was recovered from fourteen of them. Four mice presenting such symptoms and carrying virus in their blood were put in a cage by themselves and observed for 3 weeks, during which time they made a complete recovery.

The symptoms most frequently presented by naturally infected young mice cannot be regarded as pathognostic. The tremors and

TABLE I

Symptoms Presented by Naturally Infected Mice

Group	Condition of mice	No. of mice examined	Test for virus by inoculating blood or brain or both into guinea pigs	
			No. positive	No. negative
A	No symptoms; normal rate of growth	At least 13*	9	4
B	Conjunctivitis and photophobia; no other symptoms	5	2	3
C	Emaciation; ruffled fur; somnolence; slow, stiff movements; slow rate of growth	19	14	5
D	Found dead in colony	3	2	1

* The exact figure cannot be given, because in ten cases the pooled blood of from five to thirty healthy mice was injected. Each of these cases is counted as one mouse.

spastic convulsions which characterize the experimental disease in intracerebrally injected mice have not been observed in naturally occurring cases.

In Table I a list is given of mice which were examined for virus by inoculating heart blood or suspensions of brains or both intracerebrally into guinea pigs, and of the symptoms noted in the mice.

As mentioned before (1), a striking clinical picture can be produced in some mice which have a latent infection by an intracerebral injection with sterile bouillon. In 3 to 13 days after the inoculation such mice present symptoms indistinguishable from those shown by mice inoculated intracerebrally with virus: marked tremors, passing into

spastic convulsions of the hind legs when the animals are lifted by the tail.

The Experimental Disease in Mice

Intracerebral Inoculation.—In mice from the infected stock the rate of morbidity following intracerebral inoculation with virus was about 60 per cent, and the rate of mortality about 40 per cent, while in mice of the same strain which were bred from noninfected parents the rates of morbidity and mortality were practically 100 per cent. The incubation period is on the average 6 to 7 days and may vary from 5 to 12 days according to the amount of virus injected. It has never been shorter than 5 days, no matter how much virus was inoculated. On the 6th or 7th day the mice appear ill and show signs of general malaise. They are no longer lively and often sit quietly alone. Their fur is usually ruffled. When the animals are lifted by the tail, rapid motions followed by very distinct tremors of the front and hind legs result, the latter being somewhat retracted. As the disease progresses such tremors often pass into a striking spastic convulsion of the hind legs. The front legs are rarely involved in this convulsion. When the animals are dropped during the convulsion, they may lie on the side, the hind legs being stiffly stretched out, the tail rigid, and the back appearing humped. The front legs carry out a series of very rapid motions. Animals that gain the erect posture may drag themselves about the table by means of the front legs, the hind legs remaining stretched out and rigid (Fig. 1). The convulsions usually last from one to several minutes. Many mice die during the first convulsion brought about by lifting them by the tail. Others recover from the convulsion and are then able to walk about normally. Several minutes usually elapse before a second convulsion can be induced. Practically all affected mice ultimately die in convulsions, as evidenced by the position of their hind legs during rigor mortis. Death occurs in 1 to 2 days after the onset of symptoms.

Intranasal Instillation.—The intranasal instillations were carried out as follows: About 5 cc. of a 1 per cent suspension (in saline) of infective mouse brain were placed in a sterile Petri dish, and the nostrils of a slightly etherized mouse were dipped once or twice into the suspension. By this procedure a small drop of suspension became attached to the nostrils and was inhaled by the mouse immediately. In this manner the dosage could not be accurately measured, but the method is probably more similar to the natural mode of infection (if that takes place by way of the nasal passages) than the instillation of a large amount of suspension.

In the first experiment eight 6 week old mice bred from disease-free mothers were used. They were bled from the heart and their pooled defibrinated blood was inoculated into a guinea pig (0.2 cc. intracerebrally and 0.5 cc. subcutaneously into each planta), with negative result. The eight mice were then inoculated intranasally with virus. From each of two mice, 0.2 cc. blood was drawn on the 7th day after the exposure and inoculated immediately (not defibrinated) into the brain of a guinea pig. Both guinea pigs developed the disease and died on

the 14th and the 17th day, respectively, after the inoculation. Two mice were bled in the same manner on the 11th day after the exposure, and their blood was also virulent for guinea pigs. The four remaining mice were bled similarly on the 11th day and again on the 16th day. All blood samples obtained from them were avirulent for guinea pigs. None of the eight mice showed the slightest symptoms. 3 weeks after the exposure they were tested for immunity by intracerebral inoculation with virus, and the four mice which carried virus in their blood were immune; while the four others, as well as the eight normal control mice, died.

In another experiment, five half grown mice bred from disease-free parents were inoculated intranasally with virus. The animals showed no symptoms. On the 10th day after the inoculation each mouse was bled by heart puncture, and 0.2 cc. defibrinated blood was inoculated immediately into the brain of a guinea pig. None of the guinea pigs showed fever or symptoms; but one became immunized by the blood inoculation, while the four others died following the test inoculation. The mice were tested for immunity by intracerebral inoculation with virus on the 23rd day following the inoculation. One mouse, as well as six normal control mice, died; while four mice, including the one whose blood immunized a guinea pig, showed no symptoms. This result indicated that four of the five mice had become infected by the virus given intranasally.

Intraperitoneal Inoculation.—Of mice bred from virus-free, susceptible mothers about 60 per cent showed symptoms 5 to 7 days after intraperitoneal inoculation with virus. The symptoms differed from those presented by mice inoculated intracerebrally in that there was markedly labored breathing and no tremors or convulsions. The symptoms lasted for about a week, and all such mice finally recovered.

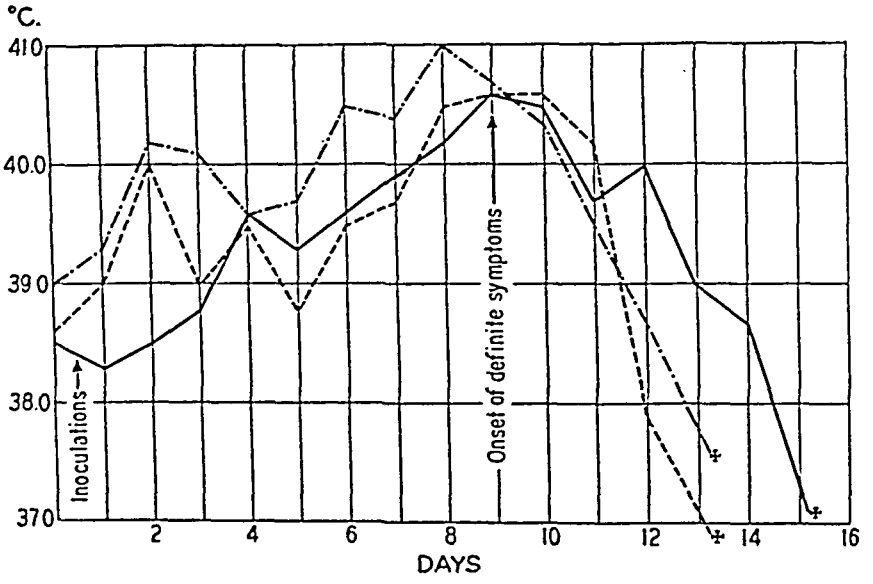
Intravenous Inoculation.—About 70 per cent of the disease-free mice injected intravenously with the supernatant fluid of a virulent guinea pig brain emulsion showed symptoms after 6 to 10 days. The symptoms were similar to those exhibited by intraperitoneally inoculated mice, except that the labored respiration was more striking. Spastic convulsions and tremors were not noted. Three out of 42 mice died; the remaining sick ones recovered slowly or were sacrificed for histological examination when they showed definite symptoms.

The Experimental Disease in Guinea Pigs

The virus has been transmitted to guinea pigs by intracerebral and subcutaneous inoculation[1] and by intranasal instillation. In a comparative titration experiment the minimal infective dose was equal for intracerebral and subcutaneous inoculation. The rates of morbidity and mortality have varied considerably with different strains

[1] All subcutaneous inoculations were made into one or both footpads in the plantar region.

of virus, but every strain produced at least a marked febrile reaction followed by a very solid immunity to a highly virulent virus. We have two strains of virus, maintained by passages through guinea pigs, which kill over 80 per cent of the guinea pigs following intracerebral and subcutaneous inoculation, and produce a very severe disease in the remaining animals. These two strains were used in neutralization tests.



TEXT-FIG. 1

—— Guinea pig inoculated intranasally with bacteria-free Berkefeld N filtrate.

—·— Guinea pig inoculated intracerebrally with mouse brain suspension.

— — — Guinea pig inoculated subcutaneously with mouse brain suspension.

The course of the disease in guinea pigs is rather slow, and the symptoms, when marked, are pathognostic. Unlike the disease in mice, there is practically no difference in the incubation period nor in the clinical picture following intracerebral, subcutaneous, and intranasal inoculations with virus. The most constant clinical feature is fever (a body temperature of 40°C. and above is considered febrile, except on hot summer days when normal guinea pigs may show temperatures of 40.5°C.). In Text-fig. 1 three typical temperature curves of experimentally infected guinea pigs are given. Other evidences of disease seldom

appear before the 9th day. There is a considerable loss of weight, and a markedly labored breathing, which may last for a few days, and gradually pass into dyspnea if the animal dies, or slowly subside if the animal recovers. In severe cases the emaciation is very pronounced, and recovering guinea pigs require a long period of time to regain the lost weight. Somnolence, salivation, and a marked seropurulent conjunctivitis are also present in severe cases. Death rarely occurs before the 12th day, and it may be delayed as long as 30 days.

The pathogenicity of a sample of the virus may vary for different guinea pigs. One animal may merely show fever and no other symptoms, while another of the same stock and the same size, inoculated with the same amount of virus at the same time, may develop a most severe, fatal disease. In all cases in which fever was the only sign of the disease, the guinea pigs were tested for immunity, usually 3 weeks after the first inoculation, by an intracerebral or subcutaneous injection with a highly virulent virus, using an adequate number of normal guinea pigs as controls.

Inoculation of Other Animals

Rats.—Five of seven white rats inoculated intracerebrally with virus died in 6 to 9 days following the inoculation. One rat became very ill on the 7th day and recovered, and one rat showed no symptoms. The clinical picture in rats was characterized by clonic-tonic spasms of the muscles of the legs and neck. The symptoms on the whole were somewhat similar to those presented by intracerebrally injected mice.

Rabbits.—Two rabbits inoculated with infective mouse brain suspension intracerebrally, intranasally, intracutaneously, intratesticularly, and by rubbing virus into the scarified corneae showed no fever and no symptoms during 1 month's observation. 3 weeks after the inoculation the rabbits were bled, and in their pooled serum neutralizing antibodies were detected. Two more rabbits were inoculated intracerebrally with infectious mouse brain suspensions. One rabbit showed no fever and no symptoms. The other had fever (41.0°C.) on the 3rd day following the inoculation. On the 4th day its temperature was again normal, and the animal never showed symptoms.

Chicks and Pigeons.—Four chicks, 6 weeks old, and two pigeons inoculated intracerebrally with virus showed no symptoms.

The Pathological Changes Produced by the Virus

Technique.—The organs examined were fixed in Zenker's fluid or, less frequently, in formalin or a combination of both. The fixed tissues were embedded in paraffin. The following staining methods were used: Iron alum hematoxylin and Van Gieson (particularly for sections of brain and spinal cord examined for changes of nerve cells), hematoxylin and eosin, Giemsa stain, eosin and methylene blue (for sections of tissues examined for acidophilic inclusion bodies), and phloxine and methylene blue. All animals (except one mouse inoculated intranasally which showed no symptoms) were killed for histological examination when they presented definite symptoms, or the organs were removed immediately after death.

Naturally Infected Mice.—The pathological changes in naturally infected mice presenting definite symptoms were scanty. Macroscopically no lesions were noted. Histologically the majority of such mice showed changes in the liver: small collections of round cells in the vicinity of blood vessels and scattered lymphocytes, single or in small groups, in the interstitial tissue. Patchy reticuloendothelial hyperplasia was frequent in the liver. In the lungs of two mice slight peribronchial and perivascular infiltrations with round cells and a slight thickening of the alveolar walls were noted. Only one of the twelve mice examined presented a slight meningitis, the exudate cells being predominantly lymphocytes. The chorioid plexuses were not involved.

Mice Inoculated Intracerebrally with Virus, and Naturally Infected Mice Inoculated Intracerebrally with Sterile Bouillon.—No definite macroscopical lesions occurred regularly in mice killed in the stage of spastic convulsions or autopsied immediately after death. In some cases the liver had a more or less pronounced nutmeg color, and the spleen was slightly enlarged. The lungs appeared normal in every case.

Microscopically a more or less marked infiltration of the meninges with round cells was noted in all of seventeen mice examined histologically. The predominating cells in the meningeal exudate were lymphocytes. Mononuclear cells were next in frequency, and a small percentage of the cells were polymorphonuclear leucocytes.[2] The meningitis was usually most intense at the base of the brain (Fig. 2). In many mice round cell infiltration was also noted in the pia-arachnoid of the spinal cord. In thirteen of the seventeen mice the chorioid plexuses were infiltrated with round cells, and in four cases the plexuses were not involved. In ten cases the infiltration was very intense. The plexuses of the third and fourth ventricles showed infiltration more frequently and more intensely than the side ventricles. In some cases many round cells had accumulated in the ventricles outside the plexuses, and in one case the third ventricle was almost completely filled with such cells. The ependyma was frequently infiltrated, and there was a moderate degree of subependymal gliosis. Very rarely the ependyma was pro-

[2] Cultures of all brains saved for histology showed no growth.

liferating. Round cell infiltrations of the perivascular lymph spaces of submeningeal and subependymal vessels were noted in ten of fifteen brains. In cross-sections of such vessels and of the vessels of the pia mater unusually large numbers of lymphocytes were often present. Some round cells in the exudate of the meninges, chorioid plexuses, and in the perivascular lymph spaces were in mitosis.

In three brains more or less large areas of the stem were infiltrated with round cells. Small collections of oligodendroglia cells were seen in a few mice in the cerebral cortex at the base of the brain. In the cerebellum of many mice small numbers of pycnotic and shrunken Purkinje cells were scattered in the rows among perfectly normal cells of this type. In the spinal cord one or two degenerated ventral horn cells surrounded by oligodendroglia and microglia cells were occasionally seen. On the whole, nerve cell degeneration and neuronophagy were not frequent, and the changes in the nervous tissue proper were few.

No inclusions were found in any types of cells.

In other organs no definite changes were noted. In several liver sections there were patchy reticuloendothelial hyperplasia and a few small collections of lymphocytes in the vicinity of blood vessels. A slight thickening of the alveolar walls in areas of the lungs and a slight bronchitis and peribronchitis (round cell collections) were noted in a few mice, but the pulmonary lesions were not nearly so marked as in guinea pigs. No changes were found in the salivary glands of mice, and it is believed that the virus is not related to the salivary gland viruses. Blood counts on a number of intracerebrally injected mice revealed no definite abnormalities.

Intranasally Injected Mice.—The organs of a mouse which had contracted a latent infection by intranasal instillation of virus were examined histologically (mouse killed on the 7th day after exposure) and no definite changes were noted. There was no evidence of meningitis or pneumonia.

Intraperitoneally Injected Mice.—In mice killed when the symptoms were very marked the spleen was often enlarged (1½ to 2 times normal size), and the liver had a nutmeg color and was slightly swollen. The lungs contained no consolidated areas. About 20 per cent of the mice autopsied presented a serous pleuritis and peritonitis, the pleural and peritoneal cavities containing an almost clear, serous exudate, films of which stained with Giemsa stain revealed a considerable number of lymphocytes and macrophages.

The organs of six mice were examined microscopically. In only one case was a slight meningitis noted; the chorioid plexuses were not involved. In all other cases the meninges appeared normal. In liver sections of all mice patchy proliferation of reticuloendothelial elements was noted, giving the section a spotted appearance when examined with lower magnification. Single or small groups of round cells were scattered throughout the interstitial tissue. More or less extensive collections of lymphocytes and mononuclears around blood vessels were frequent findings. In mice presenting serous pleuritis and peritonitis the pleural or peritoneal endothelial cells were swollen, and the pleura and peritoneum were slightly infiltrated with round cells. In the spleen the Malpighian bodies appeared

enlarged, and there was some reticuloendothelial hyperplasia. In two cases a definite interstitial pneumonia with small round cell collections around blood vessels was noted, while in four cases no definite pneumonia was present. Other organs showed no marked changes.

Blood counts on a limited number of mice injected intraperitoneally revealed no marked changes of the blood picture.

Intravenously Injected Mice.—The changes in mice showing symptoms following intravenous inoculation with virus were similar to those in intraperitoneally injected mice but more marked. In all cases the spleen was considerably enlarged, its volume being from 3 to 6 times greater than normal. The enlargement of the spleen could be readily detected in living animals by palpation. The liver appeared somewhat pale and slightly swollen. In one case one kidney was much enlarged. The lymph nodes were not definitely enlarged. A few mice presented serous pleuritis. The lungs appeared normal macroscopically. In the spleen the Malpighian bodies were enlarged, and the red pulp was more or less infiltrated with lymphocytes and mononuclear cells. The reticuloendothelial elements showed hyperplasia. In some lymph nodes reticuloendothelial hyperplasia was also noted. In the liver large round cell collections were present around the larger blood vessels, and smaller collections around the central veins and in association with proliferated Kupffer cells. Necrosis of some liver cells was noted in the immediate vicinity of the round cell aggregates. Round cell collections were also present in the kidneys, and in the case in which a marked enlargement of one kidney was noted macroscopically, huge round cell collections were present in the atrophic renal cortex and in the renal pelvis. Lung sections revealed an interstitial pneumonia in every case, and more or less extensive round cell collections around blood vessels and bronchi. In cases of pleuritis the pleural cells were swollen, and the pleura was moderately infiltrated with round cells. The endocardium and epicardium contained more or less large round cell collections. In some cases the meninges and chorioid plexuses were moderately infiltrated with round cells.

Blood counts revealed a marked increase of white blood cells (up to 55,000 per c.mm.) in three of seven cases. A more or less pronounced monocytosis (4 to 23.5 per cent) was noted in six of seven cases, and a lymphocytosis (62.5 to 80 per cent) in five cases.

Guinea Pigs.—The outstanding lesions produced by the virus in guinea pigs following intracerebral or subcutaneous inoculation or intranasal instillation are in the lungs and, less regularly, in the heart. At autopsy in an advanced stage of the disease (dyspnea) more or less extensive consolidated areas are usually present in the lungs. Such areas may be found in several lobes with no special site of predilection. In many cases lung tissue from pneumonic areas was sterile, while from others bacteria of different types were cultivated. The heart may be slightly enlarged and the ventricles slightly dilated. A few cc. of clear serous fluid were present in the thoracic cavities of some guinea pigs, and such animals usually presented a marked subcutaneous edema at the lower parts of the abdomen,

the subcutaneous tissue consisting of a thick, gelatinous mass. Some guinea pigs showed small necrotic areas of light color and rather hard consistency in the liver. The central nervous system presented nothing abnormal except a definite excess of cerebrospinal fluid in some cases.

Material from 31 guinea pigs infected by different routes was studied histologically. The dominant lesion was a typical virus pneumonia which was present in every animal sacrificed in a late stage of the disease. Lungs which appeared normal macroscopically presented pneumonic areas microscopically. A marked pulmonary edema was noted in many guinea pigs.

Heart changes, present in the majority of the guinea pigs, consisted mainly of subendothelial infiltrations with round cells. Very small, scattered round cell collections were also seen in the myocardium and in the subepicardial layer. The cardiac muscle tissue was not affected.

The infiltration of the meninges with lymphocytes and mononuclear cells (polymorphonuclear leucocytes were entirely absent) was much less intense than in mice. A meningitis was noted in four of seven guinea pigs inoculated intracerebrally with virus, and in nine of twelve guinea pigs inoculated subcutaneously. The infiltration of the chorioid plexuses was never very marked, the inflammatory cells, if present, consisting of a few lymphocytes and mononuclear cells.

In four of eight guinea pigs inoculated subcutaneously, and in four of six guinea pigs inoculated intracerebrally, intranuclear eosinophilic bodies were seen in cells of the pia mater, in mononuclear cells scattered along the meninges, and in some adventitial cells of meningeal vessels, as well as in glia cells at the periphery of the cerebral cortex. The majority of these intranuclear bodies were small, and not always so distinct as those shown in the photograph (Fig. 4). Many of them appeared as deep red, small granules, single or in small groups in a nucleus. When the bodies were as large as those in the picture, there was a definite hyperchromatosis of the nuclear membrane. In sections from five normal guinea pigs and from a number of guinea pigs inoculated with equine encephalomyelitis virus such intranuclear bodies were not found. In one of six guinea pigs inoculated intracerebrally with mouse virus, inclusion bodies were also present in a few epithelial cells of the chorioid plexus of a side ventricle. These bodies were round and stained faintly red with eosin. Because of the small number of normal brains examined, the relation of these intranuclear bodies to the mouse virus is not definitely established. Their etiological significance is doubtful, since they are absent in mice and in the lungs of guinea pigs.

Perivascular cuffs were rarely seen in sections of the brains of guinea pigs. If present, they consisted of one layer of mononuclear cells and lymphocytes. Glia proliferation was never noted, and no damage to nerve cells was detected except in a few scattered Purkinje cells which appeared pycnotic and shrunken when stained with iron hematoxylin in combination with van Gieson's method.

As a rule many liver cells showed vacuoles in the cytoplasm, and small round cell collections around blood vessels were seen in a number of liver sections. In about 20 per cent of the guinea pigs examined necrosis of groups of adjacent liver

lobules was noted. It is questionable, however, whether such necrosis was attributable to the action of the virus. In all guinea pigs presenting conjunctivitis the conjunctival epithelium was slightly infiltrated with polymorphonuclear exudate in the conjunctival sac. No definite changes were detected in the spleen, kidneys, pancreas, salivary glands, adrenals, or lymph nodes.

Rats.—Histologically a marked infiltration of the chorioid plexuses of the side ventricles with round cells was noted (Fig. 3). The meningitis was less intense than in mice but more marked than in guinea pigs.

The Distribution of the Virus in the Body of Mice and Guinea Pigs

In mice showing symptoms following intracerebral, intraperitoneal, or intravenous inoculation the virus was invariably present in the brain and blood stream, and often in the urine and nasal secretions. In guinea pigs showing definite symptoms following subcutaneous or intracerebral inoculation virus was present in the blood, brain, lung, and urine. The virus content of the brain of a subcutaneously infected guinea pig which died about a minute before blood was drawn, was higher than that of the blood.

Serological Relationship of the Virus to Other Viruses

Because of the similarity from a clinical and pathological viewpoint of the mouse virus and the virus of acute lymphocytic choriomeningitis described by Armstrong and Lillie (2), samples of immune serum prepared against the respective viruses were exchanged with Dr. Armstrong. Cross-neutralization tests revealed the serological identity of the two viruses. These tests are recorded in a paper by Armstrong and Dickens (3). Shortly after the isolation of our virus from mice, Rivers and Scott (4) obtained from two human patients suffering from acute meningitis a filtrable virus which those authors have concluded is closely related to ours and to that of Armstrong and Lillie or immunologically identical with them.

There is no similarity between this disease and other known virus diseases of mice. Infectious ectromelia (5) and spontaneous encephalitis of mice (6) are quite different both clinically and pathologically.

Tests were made to determine the relationship of this virus to those human viruses that are readily transmitted to mice. Sera of horses immune to the human or the swine influenza virus[3] failed to neutralize

[3] These sera were obtained by Dr. R. E. Shope from Dr. C. H. Andrewes of Hampstead, England.

the virus under consideration. The relationship to lymphogranuloma inguinale (climatic bubo) was tested as follows:

A guinea pig recovered from typical experimental bubos in the inguinal lymph nodes, and two mice recovered from lymphogranuloma infection following intracerebral inoculation with virus were obtained through the courtesy of Dr. A. W. Grace of the New York Hospital. They were inoculated with our virus and all succumbed, as did the normal control animals. Later seven mice which had recovered in our laboratory from lymphogranuloma infection induced by intracerebral inoculation with virus obtained from Dr. Grace, were injected intracerebrally with mouse virus. Five of them died in 7 to 8 days, and two became typically ill and recovered. All ten control mice died in 6 to 8 days. Ten mice solidly immune to intracerebral inoculation with mouse virus, and ten normal control mice were inoculated intracerebrally with lymphogranuloma virus. Of the immune mice, six became sick 8 to 14 days after the inoculation and recovered, and four showed no symptoms. Of the control mice, seven became sick at the same time and recovered, and three showed no symptoms. These experiments suggest that the mouse virus and lymphogranuloma virus do not cross-immunize. Cross-neutralization tests gave no clear cut results, because our mice are apparently not susceptible enough to lymphogranuloma, and the serum (drawn before the test inoculation) of the guinea pig immune to lymphogranuloma did not have sufficient neutralizing power on lymphogranuloma virus to permit a conclusion as to its effect on the mouse virus.

DISCUSSION

The disease caused by the virus under natural conditions in mice is mild, with a rather high rate of infection, but with a low rate of morbidity and a very low rate of mortality. Symptoms were presented by only a small percentage of young, 2 to 6 week old mice, and were not striking. The disease is not readily recognized unless transfers of material are made from infected mice to guinea pigs, or to normal mice by intracerebral inoculation.

On the other hand, mice inoculated intracerebrally with infectious material show very striking symptoms which are probably pathognostic. Diseased guinea pigs also present symptoms more or less specific for the virus. It is suggested that for diagnostic purposes intracerebral inoculations in mice and guinea pigs be carried out, and that the body temperatures of the latter be recorded daily for 2 weeks.

Different species of rodents are differently affected by intracerebral injection of this virus. Rabbits are apparently resistant. Guinea pigs develop pneumonia and a mild meningitis. White rats respond

with a marked meningitis and inflammation of the chorioid plexuses. White mice as a rule show a marked meningitis and a more or less pronounced round cell infiltration of the plexuses and ventricles, and the pulmonary lesions, if present, are usually not very marked. The symptomatology in guinea pigs, rats, and mice corresponds to the pathological picture. In the first, respiratory symptoms dominate; while in rats and mice spastic convulsions are the most striking clinical feature, for which the anatomical basis seems to be the inflammation of the meninges and chorioid plexuses and a possible overproduction of cerebrospinal fluid and consequently increased intracranial pressure.

In general, the pathological changes produced by this virus are inflammation and hyperplasia. Necrosis is very rare.

Besides serological identity, the virus of acute lymphocytic choriomeningitis and our virus have other common features, such as the striking symptoms produced by them in mice inoculated intracerebrally. The description of these symptoms given by Armstrong and Lillie (2) can be applied to mice injected intracerebrally with our virus almost word for word, and the pathological picture presented by such mice is similar to that described by Lillie (2). Both viruses are present in the brain and blood of the mice, and the pathogenicity of the viruses for laboratory animals as far as tested differs for only one species, the white rat. While Armstrong and Lillie could not infect rats, our virus caused symptoms in six of seven rats inoculated intracerebrally. It is possible that a difference in the strains of rats used was responsible for this discrepancy rather than a difference in the viruses. With both viruses intranasal instillations into mice produced no symptoms.

On the whole the evidence presented is believed to justify the conclusion that our virus is identical with the virus of acute lymphocytic choriomeningitis.

The name lymphocytic choriomeningitis does not describe the disease naturally occurring in mice, since choriomeningitis is rare in such cases. The designation is, however, adequate for the experimental disease produced in mice by intracerebral inoculation.

SUMMARY

A filtrable virus, identical with that which causes acute lymphocytic choriomeningitis, has been found to cause a disease in white mice.

Naturally infected mice usually show no symptoms, but such animals inoculated intracerebrally with sterile bouillon or other materials develop characteristic symptoms. The same symptoms are produced by intracerebral injection of the virus into mice from a disease-free stock. Guinea pigs are very susceptible and are therefore useful for detecting the virus and for neutralization tests. The disease in both naturally infected and inoculated animals is discussed and the pathological findings given.

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EXPLANATION OF PLATES

PLATE 41

FIG. 1. Mouse showing typical symptoms on the 7th day following intracerebral inoculation with virus. Spastic convulsion of hind legs after being lifted by the tail. The mouse is dragging itself about the table with the front legs.

FIG. 2. Mouse, intracerebral inoculation with virus. Marked meningitis at base of brain. Eosin and methylene blue. ×130.

PLATE 42

FIG. 3. White rat, intracerebral inoculation. Inflammation of the chorioid plexus of a side ventricle. Eosin and methylene blue. ×100.

FIG. 4. Guinea pig, intracerebral inoculation. A meningeal cell and an adventitial cell of a small meningeal blood vessel bearing eosinophilic intranuclear bodies. Eosin and methylene blue. ×2310.

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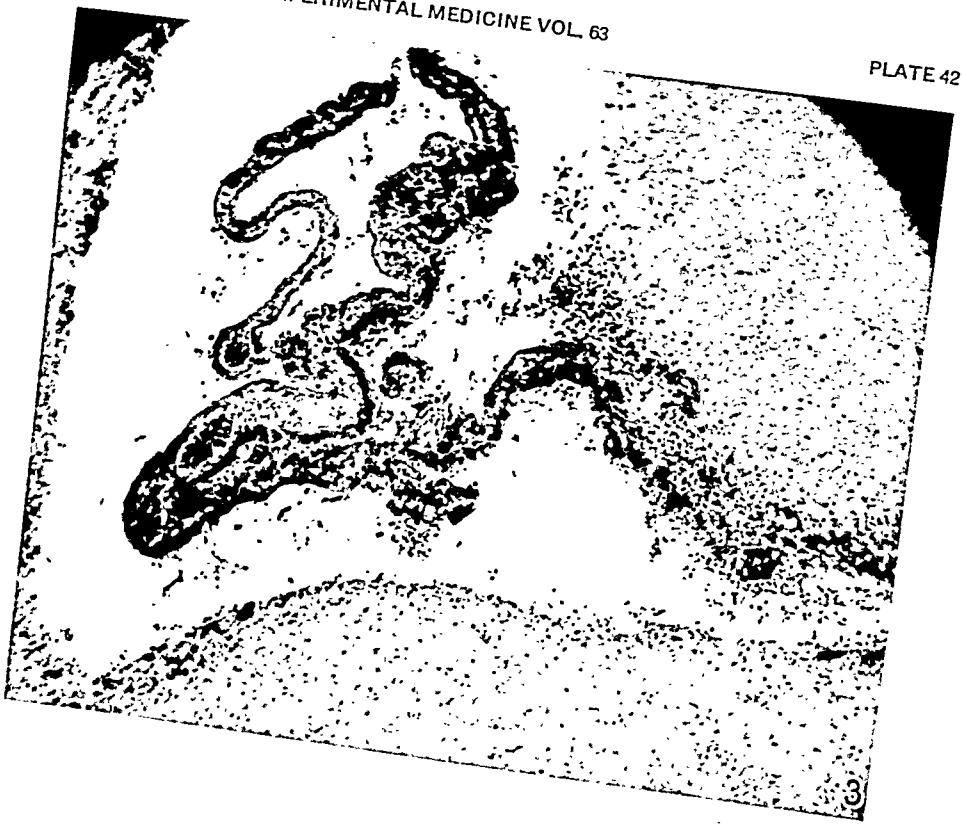

STUDIES ON MENINGOCOCCUS INFECTION

IX. Standardization and Concentration of Antimeningococcus Horse Serum (Type I)

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The problem of standardizing antisera intended for therapeutic purposes is an ever important one. In the last analysis, of course, the value of a serum must be decided by its therapeutic efficiency, but unless the relationship of the latter to some of the more conveniently measurable immunity reactions is determined, no criterion can be established whereby the probable therapeutic potency of an otherwise unknown serum may be estimated. The discovery of such a relationship depends primarily upon elucidation of the antigenic structure of the infective agent in question. For example, with Type I antipneumococcal sera, it has been possible to demonstrate a close correlation between various of the immunity reactions, in which the antibody to the type-specific polysaccharide is the factor of prime importance (1, 2). The situation has been less favorable in the case of the meningococcus, and as yet no satisfactory solution to the problem has been published.

Standardization of Type I Sera

Wadsworth (3) and Murray (4) have discussed the results which have been obtained in the past by applying various of the immunity reactions in attempts to establish a reliable measure of the potency of antimeningococcal sera. The agglutination reaction has been the most widely used and reliable method, and although the agglutinin

* Part of this work was completed by one of us while working in the laboratories of the Department of Medicine of the College of Physicians and Surgeons. The authors wish to thank Dr. Michael Heidelberger for the facilities placed at their disposal.

titre is not necessarily an index of therapeutic potency, there is evidence that sera of high titre possess therapeutic efficiency (3).

The precipitin reaction has not proved satisfactory in the past chiefly because the precipitinogen commonly used (a crude extract of meningococci) is not readily standardized and reproduced. Zozaya (5) has used what was probably the group-specific polysaccharide, or C substance, of the meningococcus as the precipitating antigen. The isolation from the Type I meningococcus of a highly purified, type-specific substance (6, 7) (to be referred to hereafter as S I) suggested the possibility of accurate measurement of the precipitin content of Type I antimeningococcal sera by the quantitative method developed by Heidelberger and Kendall (8). This has been done and in addition the method has been applied in following the fractionation and purification of such sera.

The sera used were prepared by immunizing a horse with vaccines prepared from recently isolated strains of Type I meningococcus according to the method already described (9). The S I samples used were preparations 8 and 18 previously described (7). These were found to be identical with respect to precipitating potency with horse sera.

The technique for the determination of nitrogen in the specific precipitates produced by the reaction between the sera and S I (to be referred to hereafter as antibody nitrogen[1]) followed closely that described by Heidelberger and Kendall. 1 or 2 cc. of serum or an equivalent amount of antibody solution (not more than 0.4 mg. antibody nitrogen) have been used. Since all of these contained preservative, sterile precautions were not observed. The sample was measured by a calibrated pipette into a Wassermann tube and 0.025 mg. of S I in solution was added for each cc. of serum. Duplicate tubes and salt control tubes were set up in all cases. The volume was adjusted to 4 cc. with saline and the tubes were closed with rubber caps. The contents were then mixed thoroughly and the tubes were allowed to stand for 1 or 2 hours at room temperature (20–25°C.). This procedure gave results identical with those obtained when the tubes were subjected to the customary 2 hour period of incubation at 37°C. It is realized that this method probably did not result in as complete precipitation of antibody as would have been obtained if all operations had been carried out at 0°C. (8), but this fact was not important for the purposes at hand. After the preliminary period of standing, the tubes were placed in the refrigerator and left for 18 to 24

[1] Since S I probably contains nitrogen as a constituent of its molecule, part of the antibody nitrogen may be attributable to the S I contained in the specific precipitate. However, the total amount of S I nitrogen added in one of these determinations (0.001 to 0.002 mg.) is less than the experimental error.

hours. They were then centrifuged in the cold. The specific precipitates formed compact discs which could not be readily broken up. For the first washing, the precipitate was suspended in 3 to 4 cc. of chilled saline, left for 1 hour at 0°C., and centrifuged off as before. For the second washing, it was found best to allow the precipitate to stand in contact with 3 to 4 cc. of saline at 0°C. for 16 to 24 hours with occasional shaking. In the first determinations a third washing was made, but this was found to be superfluous. Finally, the precipitate was centrifuged off in the cold. It was then dissolved in a little distilled water with the aid of a few drops of N/1 sodium hydroxide. The solution was rinsed into a 125 cc. Kjeldahl flask and the nitrogen determination was carried out according to a slight modification of the Pregl micro Kjeldahl method.

Heidelberger and Kendall (8), studying the reaction between the Type III pneumococcus polysaccharide and its antibody, found that when increasing amounts of polysaccharide were added to a fixed amount of antibody, the quantity of precipitate formed increased to a maximum and then remained constant over a wide range until the inhibition zone was reached. A similar relationship exists for the present system. It was found, with samples of serum containing between 0.15 and 0.20 mg. of specifically precipitable nitrogen, that identical results were obtained over the range 0.02 to 0.10 mg. of S I added. After removal of the specific precipitates, portions of the supernatant fluids were tested in each case (*a*) with an equal volume of antiserum, and (*b*) with an equal volume of a 1:50,000 solution of S I in saline. The (*a*) tests all showed strong reactions, whereas the (*b*) tests were uniformly negative, thus showing that S I was present in excess in all cases. Less than 0.02 mg. of S I added could not be relied upon to give maximum precipitation, whereas 0.3 mg. produced inhibition or prozone phenomenon. Since it was desired to characterize the sera by determining under standard conditions the maximum amount of specifically precipitable nitrogen which they contained, the procedure finally adopted was to add 0.025 mg. of S I (0.5 cc. of a 1:20,000 solution in saline) per cc. of serum (antibody nitrogen content, 0.1 to 0.2 mg. per cc.). These proportions were maintained throughout and were found sufficient to provide a definite excess of S I but at the same time to be remote from the inhibition zone. The supernatants from all determinations were, of course, tested qualitatively for the presence of excess S I and antibody. The sera have been analyzed for both total Kjeldahl nitrogen and

antibody nitrogen. The results are shown in Table I. Each figure represents the average of at least two determinations which checked to within less than 0.01 mg. The results are therefore believed to be accurate to within ±0.005 mg.

It will be seen that the sera used (representing successive bleedings from the same horse) contained between 1 and 2 per cent of their nitrogen in the form of antibody (0.1 to 0.2 mg. antibody nitrogen per cc.). This is rather a low figure in comparison with the results found, for example, with some antipneumococcal horse sera, where the figure may be at least ten times as great. Thus Felton (2), in a study of

TABLE I

Analysis of Type I Antimeningococcal Horse Sera

Serum No.	Antibody N per cc.	Total N per cc.	Antibody N / Total N	Remarks
	mg.	*mg.*	*per cent*	
1 + 2 pooled	0.185			Preserved with tricresol
Same after 6 mos.	0.150			
3	0.135	10.78	1.25	" " "
Same after 6 mos.	0.118			
4	0.178	13.46	1.32	" " merthiolate
Same after 6 mos.	0.174			
5	0.177	10.80	1.63	" " "
Same after 6 mos.	0.181			
6	0.106			" " "
7	0.121	12.08	1.00	" " "
8	0.109			" " "

thirty-nine sera from thirty-seven horses immunized against Types I and II pneumococcus, found that the content of antibody to the Type I specific substance varied from 0.22 to 2.36 mg. nitrogen per cc. The low figures of the present series are probably due to the antibody response characteristic of the particular horse used for immunization against Type I meningococcus, for, in the case of one polyvalent antimeningococcal horse serum tested, an antibody nitrogen content (Type I) of 0.97 mg. per cc. was observed, a figure which compares more favorably with those quoted above from Felton. It was thought that the low results might be due to a loss of precipitinogenic potency of the S I solution on prolonged storage in the refrigerator. Such was not the case, for a given solution of S I precipitated identical amounts

of antibody on two occasions, 6 months apart (sera 4 and 5). It was also possible that the S I had lost part of its precipitating power because of the process used in purifying it (7). Thus, it has been shown (10) that fragments prepared by partial hydrolysis of Type III pneumococcus polysaccharide may still exhibit precipitating action when tested with homologous horse antiserum but not with rabbit antiserum. However, even at dilutions as high as 1:2,000,000, S I precipitates homologous rabbit antiserum (7) and there is no evidence that it has been isolated in a partially hydrolyzed condition, for it gives

TABLE II

Titres of Type I Antimeningococcal Horse Sera against Various Meningococcus Antigens

Date	Serum No.	Anti-S I	Anti-C	Anti-P	Agglutination Type I	Agglutination Type II
1933						
Apr. 1	1 + 2	1:8,000,000	1:100	0	1:800	1:20
Dec. 1	3	1:8,000,000	1:1,000	1:1,000	1:800	1:100
1934						
Feb. 1	4	1:8,000,000	1:10,000	1:100	1:1,600	1:100
May 15	5	1:8,000,000	1:10,000	1:1,000	1:1,600	1:100
Oct. 1	6	1:8,000,000	1:10,000	1:100	1:3,200	1:100
Oct. 15	7	1:8,000,000	1:10,000	1:100	1:3,200	1:100
1935						
Jan. 1	8	1:8,000,000	1:10,000	1:100	1:6,400	1:100

Agglutinin titres represent dilutions of serum; the other titres represent dilutions of antigens.

no test for reducing sugars until it has been subjected to acid hydrolysis.

It is of interest in connection with the problem of preserving sera that the antibody content of sera in the presence of some preservatives may remain quantitatively constant over a protracted period of time. Thus, sera 4 and 5, which contained 0.01 per cent merthiolate,[2] showed no change in antibody content after 6 months' storage at 0–4°C. On the other hand, in a similar experiment, sera 1 + 2 pooled, and 3, which contained 0.3 per cent tricresol, showed respectively a loss of 19 per cent and 13 per cent within the same period.

[2] Supplied through the kindness of Eli Lilly and Co., Indianapolis.

In Table II are shown, for comparative purposes, the titres observed when the sera were tested against S I, against the group-specific polysaccharide, C, against the group-specific protein, P, and (for agglutination) against Types I and II meningococcus.[3] The most salient feature of these data is the relatively extremely high anti-S I titre, 1:8,000,000. The anti-C and anti-P titres remained low throughout (maximum = 10^{-4} and 10^{-3} respectively) as did the Type II agglutination titre, which was never higher than 1:100. On the other hand, the Type I agglutination titre was 1:800 at the first bleeding, and rose continuously to 1:6,400. This rise was not parallelled by an increase in the anti-S I content of the sera, and for the present such lack of correlation must remain unexplained.

Purification and Concentration of Serum

The accumulated evidence of past work indicates that the antibody in antimeningococcal horse serum is associated with the water-insoluble globulin fraction in a relationship similar to that found with antipneumococcal horse serum (11). Murdick and Cohen (12) have recently reviewed the subject and they confirm this fact. By a process of dialysis and isoelectric precipitation they were able to precipitate nearly all of the antibody (as measured by the agglutinin titre and neutralizing potency in the phenomenon of local skin reactivity (13)).

The method to be presented differs from that of the above authors chiefly in the fact that the water-insoluble globulin fraction of the horse serum has been precipitated by the use of carbon dioxide. The latter procedure is not a new one. Avery (14) has used it in fractionating antipneumococcal horse serum. He found that if a Type II serum, diluted tenfold with distilled water, was saturated with carbon dioxide, somewhat more than one-half of the protective power was found in the precipitate.

In an experiment along the lines of that of Avery, a sample of serum 3 was diluted fivefold with distilled water. Carbon dioxide was bubbled slowly through the solution at room temperature until it was obvious that a precipitate had formed. The solution was then left for 1 hour in the refrigerator. The precipitate

[3] Agglutinations were carried out for 2 hours at 37°C. and overnight in the ice box.

was collected by centrifuging. It was dissolved in a volume of 0.85 per cent sodium chloride solution equal to that of the original serum. To the clear, supernatant fluid was added sufficient solid sodium chloride to make a final concentration of 0.85 per cent. Both solutions were neutralized with N/1 sodium hydroxide and then tested qualitatively with 1:10,000 and 1:50,000 dilutions of S I.

The two solutions thus prepared reacted strongly, and it was estimated that at least one-half of the antibody present had not been precipitated.

It was next found that if the serum was subjected to dialysis before being diluted, treatment with carbon dioxide precipitated all of the antibody. The results of tests on the fractions obtained in a typical experiment using a sample of serum 3 are shown in Table III.

TABLE III

Fractionation of Type I Antimeningococcal Horse Serum by Dialysis and Precipitation with Carbon Dioxide

	Dialysis precipitate (fraction A)	CO_2 precipitate (fraction B)	CO_2 supernatant	Control, original serum
1:10,000 S I	++ pd	+++ pd	−	+++ pd
1:50,000 S I	+ p	++ pd	−	+++ pd

Readings made after 2 hours at 37°C.
pd indicates formation of disc-like precipitate easily broken up by agitation.
p indicates formation of finely divided precipitate.

On dialysis, a precipitate separated out from the serum. This was collected separately and labeled fraction A. The bulk of the serum was then diluted fivefold with distilled water and treated with carbon dioxide as above. The resultant precipitate was labeled fraction B. Each precipitate was dissolved in a volume of 0.85 per cent sodium chloride equal to that of the original serum. Sodium chloride was added to the supernatant fluid from the carbon dioxide precipitation to make a final concentration of 0.85 per cent. All solutions were neutralized with N/1 sodium hydroxide and centrifuged before being tested with S I.

It will be seen that fraction A contained a considerable amount of antibody and that fraction B reacted almost as strongly as did the original serum. The most important fact was that the supernatant fluid after the removal of fraction B failed to react, indicating that the antibody had been precipitated quantitatively. This was sub-

stantiated in a similar experiment in which 100 cc. of serum 3 was fractionated. This represented 13.5 mg. of antibody nitrogen. Fraction A was found to contain 4.58 mg. (34 per cent) of the antibody nitrogen, whereas fraction B contained 8.55 mg. (63 per cent). The total recovery was therefore 97 per cent.

On the basis of these findings, the following method has been evolved for the purification and concentration of Type I antimeningococcal horse serum.

Portions of 100 to 400 cc. of serum have been used, both with and without preservative (0.3 per cent tricresol or 0.01 per cent merthiolate). The serum was placed in a bag made of either cellophane or collodion and dialyzed against cold running tap water for 24 hours. Serum which contained no preservative was protected against contamination by the use of toluene or chloroform. The precipitate which separated out as a result of dialysis was collected by centrifuging and treated as below. The supernatant was set aside for the carbon dioxide precipitation.

The dialysis precipitate was taken up in a volume of 0.85 per cent sodium chloride[4] equal to 1/15 to 1/30 the volume of the original serum. The volume chosen depended upon the extent to which it was desired to concentrate the antibody. The former quantity ensured easier manipulation and gave a final product having a volume 1/3 to 1/4 that of the original serum. The solution of the dialysis precipitate was usually very cloudy and could not be satisfactorily clarified by centrifuging or increasing the salt content of the solution. This effect was especially pronounced in those cases where tricresol had been used as preservative. It was found that the addition of 0.5 to 2 volumes of distilled water to the solution would cause the formation of a precipitate consisting mostly of inactive material. When this precipitate was centrifuged off, the supernatant was quite clear and entirely satisfactory even for precipitin ring tests, where a slight cloudiness in the serum makes reading difficult. The exact proportion of distilled water necessary to throw out this inactive material from solution varied from one preparation to another and depended particularly on the protein concentration. Thus, the smaller the volume of saline used for dissolving the dialysis precipitate, the larger had to be the proportion of distilled water added. In general, not less than 0.5 volume nor more than 2 volumes was required. Care must be exercised in carrying out this step, for the use of an excess of distilled water caused precipitation and denaturation of the antibody, which then proved to be insoluble in saline and had to be discarded.

The bulk of the dialyzed serum was diluted to five times the volume of the original serum with distilled water saturated with chloroform to prevent bacterial

[4] All solutions contained 0.01 per cent merthiolate, except the distilled water used in diluting the dialyzed serum preparatory to the carbon dioxide precipitation.

contamination. A slow current of carbon dioxide was passed through the solution until aggregates of precipitate formed which were readily visible. The carbon dioxide treatment was continued for 15 minutes to ensure the presence of an excess of the gas. The precipitate could be centrifuged off immediately without serious loss of antibody from incomplete precipitation. It separated as a compact gum which was rather difficult to manipulate.

TABLE IV

Analysis of Type I Meningococcus Antibody Preparations

Preparation No.	Volume serum taken	Volume antibody solution	Total N per cc. of antibody solution	Antibody N per cc. of antibody solution	Yield total N	Yield antibody N	Purification factor*	Remarks
	cc.	cc.	mg.	mg.	per cent	per cent		
7	200 No. 3 + 250 No. 4	64	5.12	0.880	5.9	79	13.5	Filtered through Seitz EK
8	400 No. 3	52	6.45	0.762	7.8	73	9.5	" "
9	200 No. 5	52	3.59	0.600	8.6	89	10.3	Tricresolized serum
10	50 No. 3† + 50 No. 7	58.5	2.08	0.163	10.6	80	7.5	Not filtered " "
11	45 pooled Nos. 1 to 8‡	33	1.34	0.144	8.7	91	10.4	" "

* Antibody nitrogen yield divided by total nitrogen yield.

† Had been stored 6 months. Antibody N content had fallen to 0.118 mg. per cc.

‡ Contained 11.24 mg. total N per cc. and 0.116 mg. antibody N per cc.

The solution of the dialysis precipitate was diluted with 1/10 its volume of M/1 phosphate buffer, pH 7.2, in order to increase the salt content to normal and to neutralize the solution. This solution was then used to dissolve the fraction precipitated by carbon dioxide. The final solution of the product was usually clear, colorless, and only slightly opalescent. On standing at 0 to 4°C., a small amount of precipitate formed slowly. This process has been accelerated by incubating the antibody solution for several hours at 37°C. and then allowing it to stand in the refrigerator for several days, after which the precipitate may be centrifuged off.

The antibody solutions have been analyzed for total Kjeldahl nitrogen and for antibody nitrogen according to the technique outlined. The analyses (Table IV) show that a 90 per cent yield of antibody was obtainable, while the corresponding purification with respect to total nitrogen averaged at least tenfold. The relatively low yields in preparations 7 and 8 are undoubtedly due to adsorption taking place during filtration through a Seitz filter. Since the purification factors remained relatively unchanged, it is probable that both specific and nonspecific proteins were alike lost. In preparation 10, the serum, after the treatment with carbon dioxide, was left in the refrigerator for 24 hours. The precipitate collected on the walls of the flask, permitting

TABLE V

Name or number	Type I serum	Type I serum concentrated	Type II serum	Type II serum concentrated	Agglutination	Notes
C.	+	++	0	±	I-III	Before intrathecal serum
W.	0	0	0	0	I-III	After intrathecal serum
505	++	+++	∓	±	I-III	
506	++	+++	0	±	I-III	
F.	0	±	±	+	II	
504	0	±	+	++	II	
498	0	±	±	+	II*	
500	0	∓	±	+	II*	
I	0	±	0	±	Pneumococcus 10	

II* = atypical Type II strain (15).

decantation of the clear supernatant, which was subsequently found to contain no antibody. It is obvious from the low purification factor (7.5) that a considerable amount of inactive protein separated out during this treatment. The low yield was found to be due to the use of too large a proportion of distilled water in the purification of the dialysis precipitate. In preparation 11, the fraction precipitated by carbon dioxide was centrifuged off immediately after it formed and the supernatant was placed in the refrigerator for 48 hours. A further precipitate formed which upon analysis was found to contain only 0.15 mg. or 3 per cent of the original antibody nitrogen and 9.74 mg. or 2 per cent of the original total nitrogen. In other words, the anti-

body purification obtained in this additional fraction was so slight in comparison with that obtained in the main fractions, that it was not considered worth while to collect it. The optimum procedure, therefore, is to collect the fraction precipitated by carbon dioxide at room temperature as soon as it is formed.

It had been hoped that sera concentrated in this manner could be used in the precipitin test with specimens of spinal fluid from cases of cerebrospinal meningitis sent into the laboratory to be typed. Such sera might be of especial value in the case of those spinal fluids whose content of precipitinogen was too low to react with the unconcentrated sera. However, even when trouble caused by the cloudiness of early preparations of the concentrated sera had been overcome, the results were confused by the occurrence of heterologous reactions. This is brought out by the data summarized in Table V, which also illustrates the degree of reaction to be expected in testing spinal fluids. The reactions with the concentrated sera were stronger throughout, and despite the cross-reactions observed, a correct diagnosis could have been made in all cases. It is probable that preliminary absorption of the sera with the group-specific antigen of the meningococcus would eliminate these troublesome cross-reactions.

DISCUSSION

The quantitative determination of precipitins is the most accurate method available for the standardization of sera. Whether the type-specific precipitin content of an antimeningococcal serum is an indication of its therapeutic value has yet to be studied by protection tests in experimental meningococcus infection in mice (16) and in possible clinical applications to man. For the present, it can be said that of the components thus far identified in the antigenic complex of the meningococcus, the type-specific substance elicits the most prompt and the greatest antibody response (9, 17), at least when the vaccines used have been made from freshly isolated strains of the organism. Contrary to what might have been expected, the agglutination titre has not run parallel to the type-specific precipitin content of the sera nor to the precipitin titre of the sera with respect to the group-specific polysaccharide and protein of the meningococcus. Whether this fact is due to partial degradation of the haptenes as isolated, or indicates the existence of haptenes as yet unidentified, cannot be said. It must

be borne in mind that the data presented represent the antibody response of a single horse and so are not subject to broad generalization.

The use of carbon dioxide as a precipitating agent in the purification and concentration of antibodies seems to offer certain advantages. All operations may be conducted at room temperature (20–25°C.). The precipitation is rapid, nearly quantitative, and not critical as to optimum conditions. There is no problem concerning removal of the precipitating agent, as is the case when salting-out methods are used. Type II antimeningococcal horse sera have been concentrated by the method outlined and it is not unlikely that the latter may find application in the purification of other types of antibody.

SUMMARY

Type I antimeningococcal horse sera have been standardized by the quantitative determination of their type-specific precipitin content.

By a method involving dialysis and precipitation by treatment with carbon dioxide, the antibody in such sera has been purified tenfold with respect to the nitrogen content.

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STUDIES ON THE COMMON COLD

VI. Cultivation of the Virus in Tissue Medium

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The studies of Kruse (1) in 1914 and of Foster (2) in 1916 indicated that the common cold is caused by a filtrable virus. Subsequent investigations by later workers, although confirmatory in part, developed a doubt concerning the validity of Kruse's hypothesis. After a preliminary study of the relationship of the ordinary bacteria of the respiratory tract to common cold we have come to the conclusion that a filtrable virus is in fact the principal causative agent of this disease. This conclusion is based upon the observation that it is possible to produce experimentally typical colds in anthropoid apes and in human volunteers by intranasal inoculations of bacteria-free filtrates of nasopharyngeal washings derived from individuals in the acute stage of a common cold (3). The experimental production of colds in human beings by the methods described has been confirmed by other investigators (4).

In previously published studies we have reported the successful cultivation of the virus of the common cold in tissue medium containing living chick embryo (5). Foster (6) using media containing rabbit kidney, had previously reported a similar result and it has lately been confirmed by Powell and Clowes (7). Since the publication of these early studies our experience has been considerably extended both as to the methods of cultivation of this virus and as to the character of its activity. The details of this experience are set forth in the present paper.

Procedure

1. Method of Obtaining Virus Containing Material from Patients.—The following criteria are used in the selection of a suitable individual, judged to be suffering

from common cold: the presence of an acute infection of the upper respirat tract characterized by profuse catarrhal symptoms in an individual not subj to allergic rhinitis or recurrent sinus disease; the infection should run a cou typical of the common cold; the absence of fever, great prostration, and marl inflammation of the pharynx and tonsils. The patient selected must have b free from symptoms referable to the upper respiratory tract for a considera period of time before the onset of the current infection and preferably giv history of exposure to an infection of similar nature. Nasopharyngeal washi from such a patient are collected 18 to 30 hours after the onset of the cold by stilling into the nares in small amounts about 30 cc. of NaCl-free bouillon ma from non-toxic casein peptone and containing 0.1 per cent gelatin. The pati also gargles an additional 30 cc. in divided portions. The products of the na pharyngeal washing and the gargling are combined, shaken thoroughly with gl beads and passed through a Seitz filter at 20 pounds pressure. With a few exc tions noted in the ten protocols below, the filtrate is concentrated by vacu distillation at 38°C. to approximately one-sixth its original volume. In order test for the presence of visible, cultivable bacteria, the filtrate is cultured on blo plates which are incubated both aerobically and anaerobically.

2. Technique of in Vitro Cultivation of the Virus.—The medium is prepa under a dust-proof hood; fertile eggs incubated from 9 to 11 days are opened af preliminary cleansing with alcohol, and the embryo removed to a watch-gla where, after washing with broth, it is minced with a scissors. The minced tis is pipetted into culture tubes having a diameter of 2 cm., approximately one-h of a 10 day embryo being placed in each tube. 10 cc. of the special peptone bro described are then added, together with enough cysteine hydrochloride[1] to bri the final concentration to 1:2,000. The tubes are then covered with a hea vaseline seal and kept in the ice box at 4°C. until used.

Studies of the time of survival of embryonic tissue cells under the conditions the experiment have been made. Explants[2] upon media suitable for the *in vi* cultivation of living tissue have been prepared. After 9 days storage of the m dium at 4°C. 88 per cent of the explants grew actively. The percentage of positi explants after 9 days storage was exactly the same as that observed when t explants were made from fresh chick embryo, indicating a satisfactory survi of the embryonic cells of the medium under the conditions of storage. At inc bator temperature of 37°C. the death of the embryonic cells of the medium is mo rapid. After 24 hours incubation satisfactory viability of the cells is still o served, but after 48 hours at 37°C. viability can no longer be demonstrated. culture is initiated by planting 1 cc. of virus containing material through the va line seal, and anaerobic conditions are restored by remelting the vaseline se Transfers of the culture are made after incubation at 37°C. by pipetting 1 cc.

[1] Made up in 1 per cent solution, neutralized with sodium hydroxide, steriliz by autoclaving, and kept under vaseline seal.

[2] These explants were prepared through the courtesy of Dr. Thomas M. Rive

a new culture tube. As will be seen from the following protocols, in the early stages of the work transfers were made at intervals of 5 to 9 days. As time has gone on, however, the importance of maintaining the virulence of the culture by more frequent transfer has been made manifest to us, so that cultures are now transferred every 48 hours. Reference to this point will again be made later on.

Elaborate precautions against loss of the culture through contamination must be observed. Our routine procedure is as follows: Embryos are minced one at a time, fresh glassware and instruments (sterilized by dry heat) being used for each. After two tubes have been prepared from an embryo, a little of the embryonic residue is put in a separate smaller tube of culture medium and incubated for 24 hours. Three successive aerobic blood plate cultures are made from this at intervals of 24 hours, and should a contaminant appear the two corresponding tubes of culture medium are discarded. Each strain of virus under cultivation is carried in eight tubes of culture medium. As each tube is opened for transfer a smear is made for microscopic examination, together with an aerobic and an anaerobic blood plate. Should a contaminant appear, the appropriate tube is discarded. These precautions are deemed necessary because of the occasional appearance of a slow growing diphtheroid, an anaerobe which is capable of becoming by adaptation a facultative aerobe. This organism appears to come from the egg, and cannot always be detected amidst the debris of the minced tissue in a stained smear made from a contaminated culture. Staphylococci, streptococci, and *B. subtilis* are infrequent contaminants and are easily detected. Before use for experimental purposes, each culture strain is injected intracerebrally into a rabbit to rule out the presence of herpes virus.[3]

3. Selection of Volunteers for Transmission Experiments.—Males between the ages of 21 and 60 who give no history of sinus infection, rheumatic fever, or tuberculosis are selected. The nose and throat must show no evidence of recent infection and the general physical examination must be negative. At least 6 weeks freedom from symptoms of upper respiratory infection is required. By means of preliminary nose and throat cultures and mouse inoculation of sputum, all carriers of Pneumococcus Types I, II, or III, or hemolytic streptococcus are excluded from the tests.

4. Technique of Isolation.—All experiments are conducted in a private room in Harkness Pavilion. No visitors are permitted except the special nurse in charge of the patients and the physician in charge of the experiment. Such individuals when in the room wear sterile masks, operating gowns, and rubber gloves. The food is cleanly prepared by the nurse in charge, but not sterilized. No attempt is made to sterilize the floor. A minimum of 48 hours preliminary observation is carried out in all experiments. This isolation technique appears adequate since no spontaneous infection has ever occurred.

[3] It is possible that the anaerobic technique facilitates the inauguration of growth by slight injury to the embryonic cells, thus diminishing their resistance to invasion by the virus.

5. *Inoculation of the Culture to Be Tested.*—The volunteer lies on his back, and between 1.0 and 1.5 cc. of the inoculum is permitted to run slowly up each nostril. The volunteer then turns on his face for a period of 1 minute. Two inoculations of this type are made at intervals of about 5 hours. As a rule, each culture to be tested is tried simultaneously on 3 volunteers. No experiments have been performed in order to determine the minimal infective dose of a culture of virus of common cold.

6. *Clinical Observation of the Inoculated Individual.*—The physician in charge of the experiment observes and questions the subject frequently during the study and a trained nurse notes the objective manifestations of respiratory infection which occur during the day. Her notes are made when the volunteer is off guard and are helpful in evaluating the symptoms of the individual who overstates or understates his complaints. At times it has been found helpful to convince the volunteer that the inoculated material is expected to be inert.

7. *Bacteriological Observations.*—Daily cultures of the nose and throat are usually made throughout the volunteer's stay in the hospital.

8. *Preparation of the Virus Growing in Tissue Culture for Intranasal Inoculation of Volunteers.*—The contents of a tube is transferred to a sterile mortar, and the minced embryonic tissue is ground up without sand. The supernatant fluid is used for inoculation after centrifuging 3 minutes at 1,000 R.P.M.

Description of the Experimental Cold

The experimental cold of average severity has an incubation period of about 10 hours. A volunteer who receives his first inoculation at noon usually notes some dryness of the throat and heaviness of the head before going to bed; symptoms of coryza, in individuals successfully inoculated, regularly appear the following morning. This incubation period is remarkably constant except in the case of cultures which have been frequently transferred and are falling off in potency, when it is likely to be prolonged. Symptoms usually increase in severity for 48 hours, and then begin to subside, so that the individual is nearly restored to normal at the end of 4 or 5 days. Recrudescences after discharge from the hospital are not uncommon, however.

As a rule, symptoms conform to the familiar pattern of the common cold—*i.e.* sneezing and congestion of the nasal passages at onset, with a discharge which thickens later so as to cause obstructed breathing. There is usually a productive cough. Constitutional symptoms are not infrequently noted but fever has never been observed after inoculation of virus of common cold. The throat shows increased redness and the solitary lymph follicles are enlarged, but severe sore

The above experiment indicates that the common cold virus when stored at 4°C. retains its activity for at least 14 days, for in every instance of inoculation a cold was produced. Four of these colds were of average severity, and one was of more than average severity. Furthermore, the experiment indicates that cold virus can be passed successfully from one individual to another in series, the virus used for infection of volunteer 11 having been passed serially and produced experimental infections in volunteers 6 and 8.

Strain W, the first successful culture of the virus of common cold in tissue medium, was obtained in November, 1930, from volunteer 11, whose record appears in Experiment 2. The inoculation of volunteer 11 represented the 3rd serial passage in human beings of a strain of virus obtained from a spontaneous human cold. Nasopharyngeal washings were prepared from volunteer 11, 18 hours after the onset of symptoms. The washings were filtered though a Seitz filter concentrated 7 times by vacuum distillation and preserved for 5 days at

Experiment 3

Strain W Transfers Made at Intervals of 5 to 9 Days

Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
12	1st	0	0	
14	1st	± 3 days	0	
16	6th	++ 5 "	0	
17	6th	0	0	
18	6th	++ 5 days	0	Coryza
19	10th		0	
20	10th	0		Nasal obstruction, nasal discharge, cough
21	10th	0	0	
22	12th	0	0	
23	12th	± 3 days	0	
24	12th	0	0	
27	15th	0	0	Slight coryza
28	15th	++++ 9 days	+ 4 days	Nasal obstruction, purulent bloody discharge, productive cough, sinusitis
29	15th	+++ 9 "	+ 3 "	Nasal obstruction, nasal discharge, productive cough
32	25th	0	0	
33	25th	0	0	
		+ 7 days	0	
34	25th	0	0	Nasal obstruction, nasal discharge, productive cough, headache

Experiment 1

October, 1930. Unconcentrated Virus Containing Washings Preserved 2 Weeks at Room Temperature

Volunteer No.	Filtrate	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
2	S	±* 3 days	0	Nasal obstruction, productive cough, red throat
3	B	+ 4 "	0	Nasal obstruction, productive cough, red throat
4	C	0	0	
5	D	0	0	

* ± indicates mild symptoms; + denotes a definite cold; ++ indicates a cold of average severity; +++ of more than average severity; ++++ indicates an extremely severe cold.

In the next series of tests the virus containing filtrates were preserved at ice box temperature for from 4 to 13 days. Five filtrates from different individuals were preserved in this manner and were inoculated intranasally into 5 separate volunteers. In all but one instance (filtrate H) the filtrates were concentrated by vacuum distillation at 38°C. Volunteers 6, 8, and 11 were used for the passage of the virus in series; volunteer 6 received filtered nasopharyngeal washings from a spontaneous human cold, volunteer 8 the filtered washings from volunteer 6 at the height of his symptoms, and volunteer 11, those from volunteer 8 taken under similar conditions. The results of these tests are shown in Experiment 2.

Experiment 2

Concentrated Virus Containing Washings Preserved 4 to 13 Days at Ice Box Temperature

Volunteer No.	Filtrate	Duration and intensity of symptoms		Remarks
		Respiratory	Constitutional	
6	H	++ 3 days	0	Nasal obstruction and discharge, productive cough, red throat, headache
8	6	++ 4 "	0	Marked coryza, red throat
9	DP	++ 4 "	0	Coryza, productive cough, red throat, headache
10	I	+++ 4 "	+ 2 days	Coryza, productive cough, red sore throat, headache, anorexia, malaise, sweating
11	S	++ 4 "	+ 1 day	Coryza, cough, anorexia, malaise, sweating, red throat

In April, 1931, strain M was cultivated in a manner similar to strain W from a patient suffering from a typical common cold. The infectivity of this strain (culture transfer 17) was tested on 3 human volunteers. The results are shown in Experiment 4.

Experiment 4

Strain M Transfers Made at Intervals of 4 to 5 Days

Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
35	17th	0	±	Headache, anorexia, vomiting, red edematous throat, postnasal discharge
36	17th	0	0	
37	17th	++ 5 days	+	Nasal obstruction, nasal discharge, productive cough, headache, malaise, anorexia, vomiting, red edematous throat, postnasal discharge

As a consequence of the above series of inoculations volunteer 37 experienced an acute head cold of average severity associated with a moderate degree of constitutional reaction. Volunteer 35 developed no respiratory symptoms and showed constitutional reaction only consisting of listlessness, malaise, and vomiting. Volunteer 36 remained free from symptoms. The above experiment indicates the successful cultivation of the virus of the common cold in chick embryo medium for 17 transfers, a total period of 76 days.

Cultures of strain J when inoculated into human volunteers produced experimental common colds by the use of transfers 2, 19, and 50. Of a total number of 11 tests, 5 gave positive and 6 negative results. In this culture cold virus was propagated in the tissue medium for at least 50 transfers and for a total period of time of 159 days.

In Experiment 6 an effort was made to determine whether the virus of common cold could be successfully cultivated under aerobic conditions.

The medium used was in every way similar to that used in the anaerobic technique with the exception that no cysteine hydrochloride was added and the cul-

ice box temperature in order to permit the testing of the filtrate for the presence of visible bacteria. After the lapse of this interval 0.25 cc. of the concentrated, bacteria-free material was inoculated into tissue medium of the nature described. The results of the inoculation of human volunteers with the various transfers of this culture are shown in Experiment 3.

In order to test the communicability of the experimental colds produced by culture virus, nasopharyngeal washings were obtained from volunteers 27 and 28 infected with the 15th transfer of strain W. These washings were prepared on the day of infection when the symptoms were well developed and Seitz filtrates were inoculated intranasally into 2 volunteers who were in waiting for the occasion. The results are shown in the following protocol.

Volunteer No.	Passage virus	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
30	From volunteer 27	± 2 days	0	Slight coryza
31	From volunteer 28	+ 3 days	0	Nasal obstruction, slight bloody discharge, productive cough, headache

Experiment 3 indicates that the common cold virus has been propagated outside the human body by cultivation in chick embyo medium for at least 25 successive transfers involving a total period of 107 days. Volunteers were successfully inoculated with transfers 1, 6, 12, 15, and 25.

The experimental colds varied in intensity from a mild symptomatology to one of great severity. The severest colds were produced with the 15th transfer and both showed complications—one sinusitis and the other bronchitis. The reason for the variability in severity of colds developing from inoculation of the different transfers is obscure and may be due either to varying susceptibility to infection of the volunteers or to changes in infectivity of the virus. In all, six cultures of strain W were inoculated into 17 volunteers, of whom 7 manifested symptoms of the common cold and 10 remained free from infection. From volunteers 27 and 28, who received the 15th transfer of strain W, filtered nasopharyngeal washings were obtained and 2 additional volunteers were infected by the intranasal instillation of these filtrates, indicating that an experimental cold induced by culture virus can be passed in series to susceptible human beings.

Strain J used for the inoculation of 6 volunteers in Experiment 6 had been cultivated for 21 transfers under aerobic conditions. None of the volunteers inoculated developed respiratory symptoms of sufficient definiteness to justify the conclusion that an experimental cold had been produced by inoculation of an aerobic culture of strain J. The question mark opposite volunteer 50 indicates that he developed slight symptoms for 2 days following inoculation which were not, however, of sufficient magnitude to designate them as indicating a positive result. Experiment 5 shows that the 50th transfer of strain J cultivated under anaerobic conditions was capable of producing a severe upper respiratory infection in one volunteer and a mild infection in another. The above experiment would seem therefore to show that aerobic conditions of cultivation are not favorable to the maintenance of infectivity by the virus of common cold, the virus either failing to grow under these conditions or else falling off in virulence to such a degree that it is no longer capable of producing experimental infection.

Experiment 7

Strain K. The culture was isolated in October, 1932, from a patient on the 2nd day of a typical cold. Transfers were made at intervals of 3 to 4 days.

Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
71	34th	0	0	
72	34th	++ 5 days	0	Moderate coryza, productive cough

The 34th transfer of strain K when inoculated into 2 human volunteers produced in one of them a typical cold of moderate severity. The virus was active after 124 days of cultivation.

Inoculation of the 19th transfer of strain P produced no experimental infection in any of 3 volunteers. The technique of isolation and cultivation of the virus was the same as that used in previous experiments. Although a single series of inoculations is not sufficient proof of the activity or inactivity of a culture, Experiment 8 must be regarded as illustrating a failure to cultivate the virus of common cold.

Experiment 5

Culture strain J was isolated in September, 1931, in a manner similar to that of the two preceding cultures. Transfers were made at intervals of 3 to 5 days.

Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
38	2nd	0	0	
39	2nd	+ 5 days	0	Moderate coryza, headache
40	2nd	0	0	
41	15th	0	0	
42	15th	0	0	
43	15th	0	0	
44	19th	++ 4 days	+ 1 day	Nasal obstruction, nasal discharge, productive cough, red edematous throat, headache, malaise
45	19th	0	0	
46	19th	+ 4 days	0	Moderate coryza, productive cough, red throat
47	50th	± 4 "	0	Slight coryza productive cough
48	50th	+++ 4 "	+ 1 day	Nasal obstruction and discharge, productive cough, red edematous throat, headache, malaise

tures were not sealed with vaseline. Strain J was used for the purpose and anaerobic and aerobic cultures derived from transfer 29 were carried along under exactly similar conditions. The 50th transfer of the anaerobic culture was tested and the results are shown in Experiment 5. The 49th and 50th transfers of the culture, representing the 20th and 21st aerobic transfer, were tested in human volunteers and the results are shown in Experiment 6.

Experiment 6

Strain J. Cultivated under Aerobic Conditions

Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
49	49th + 50th (mixed)	0	0	
50	49th + 50th (mixed)	? 0	0	Very slight coryzal symptoms
51	49th + 50th (mixed)	0	0	
52	50th	0	0	
53	50th	0	0	
54	50th	0	0	

previously stated chick embryo tissue kept under strictly anaerobic conditions at incubator temperature is probably dead after the lapse of about 36 hours. This is indicated by the fact that after this length of time no positive explants on medium suitable for the cultivation of tissue can be obtained. We have believed that dead degenerating tissue cells would exert an injurious influence on the growing virus and that if it were protected as far as possible against such effect the virulence might be maintained for longer periods of time. That this assumption seems to be true is shown in the following experiments.

Experiment 10

Strain Ro. The culture was isolated in October, 1934, from a typical cold on the 2nd day of symptoms. The transfers were made at intervals of 48 and 72 hours. Total duration of cultivation 343 days.

Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
101	13th	+ 4 days	0	Moderate coryza, headache
102	13th	+++ 7 "	+ 3 days	Severe coryza, productive cough, headache, anorexia, malaise
103	13th	+++ 7 "	+ 3 "	Nasal discharge, productive cough, malaise, sore throat
107	75th	++ 7 "	+ 5 "	Marked coryza, cough, sore red throat, headache, anorexia, malaise
108	75th	+++ 6 "	0	Marked coryza, slight cough, sore red throat, headache
109	75th	0	0	
110	88th	+++ 7 days	0	Marked coryza, moderate cough, sore red throat, headache
111	88th	++ 6 "	0	Nasal obstruction and discharge, productive cough
112	88th	+++ 7 "	+ 6 days	Marked coryza, productive cough, sore red throat, severe headache
113	135th	0	0	
114	135th	±	0	Sore throat, headache
115	144th	±	0	Nasal obstruction, slight sore throat, cough
116	144th	±	0	Slight coryza, cough

Experiment 8

Strain P. The culture was isolated in November, 1933, from a common co of uncertain duration and was transferred at 3 to 4 day intervals.

Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
85	19th	0	0	
86	19th	0	0	
87	19th	0	0	

Experiment 9

Strain T. The culture was isolated in February, 1934, from a typical common cold on the first day and was transferred at 2 to 4 day intervals. The period of cultivation extended from April to November, 1934.

Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
91	17th	0	0	
92	17th	++ 4 days	+ 1 day	Coryza, productive cough, headache, anorexia, malaise, red throat
101	81st		0	
102	81st		0	
103	81st		0	

Inoculation of the 17th transfer of culture strain T produced an experimental cold of average severity in one of 2 volunteers. The 81st transfer of this culture was inoculated into 3 volunteers but no experimental infections resulted from the inoculations, indicating that the virus at the end of 81 transfers was either dead or had lost its infectivity. The volunteers used for the inoculation of transfer 81 of strain T were shown 72 hours later to be susceptible to an experimental cold by the inoculation of a recently isolated strain R of cold virus following which all 3 developed typical colds. Our experience leads us to believe that the cultivated virus of common cold loses its infectivity for human beings after a certain period of cultivation *in vitro* but that in all probability the virus continues to grow outside the body for an indefinite length of time.

The two cultures of cold virus described in the following experiments have been transferred at 48 hour intervals. As has been

For purposes of illustration the protocol of a typical positive infe
tion with culture virus of common cold is inserted at this point.

Protocol 1.—Volunteer 118. Nov. 7, 1935. This is volunteer 118's sixth ad-
mission to the isolation quarters. His previous record appears under Nos. 82,
103, 105, 110, and 113 in this and a later paper on the cultivation of a filtrable
virus from examples of human influenza. He ordinarily experiences from two
to three colds a year. He has had no recent colds and in September, 1935, ex-
perienced no infection following inoculation with strain Ro 135 dried with gum
acacia. On examination he showed no signs of recent infection of the respiratory
tract. He was first inoculated with strain Ce 126, a tissue medium culture from
a patient with influenza. This inoculation proved negative. He was later inocu-

TABLE I

Strains W, M, J, K, and T. Transfers at 3 to 9 day intervals		
Total No. of inoculations	38	*per cent*
No. of negative results	23	
Infections indicated as ± to ++	12	60
Infections indicated as +++ or more	3	32
Total No. of positive infections	15	8
		40
Strains Ro and Wh. Transfers at 48 and 72 hr. intervals		
Total No. of inoculations	12	
No. of negative results	2	
Infections indicated as ± to ++	3	17
Infections indicated as +++ or more	7	25
Total No. of positive infections	10	58
		83

lated with strain Wh 14, a freshly isolated culture from a patient with common
cold. The culture had been transferred at 2 day intervals. Nov. 8. Complains of no
symptoms. Nov. 7, 1935. Admitted to isolation quarters. At 12 noon received 1.5 cc.
Ce 126 in each naris. 5 p.m. received 1.5 cc. Ce 126 in each naris. Nov. 10–14.
No signs or symptoms whatever have resulted from this inoculation. Nov. 14.
Throat appears as on admission. 12 noon received 1.5 cc. Wh 14 in each naris,
5 p.m. received 1.5 cc. Wh 14 in each naris. Nov. 15. His first symptom, cough,
began at 9:30 p.m. on the evening of inoculation. This morning he has nasal
obstruction with a watery coryza. Symptoms became increasingly severe during
the day. Nov. 16. He has nasal obstruction and a mucoid discharge from the
nose. A severe productive cough is present. In the afternoon some malaise
and anorexia appeared. He was given an ephedrin spray and a mixture contain-

Study of the above table indicates that when a culture of common cold virus is transferred at 48 and 72 hour intervals the virulence and infectivity seem to be well maintained for a considerable period of time and a high proportion of relatively severe infections result from the inoculation of human volunteers. Strain Ro maintained a high degree of virulence for 88 transfers, a total period of 219 days.

Experiment 11

Strain Wh. This culture was isolated in September, 1935, from a typical common cold on the first day of symptoms. Transfers were made at 48 and 72 hour intervals. The period of cultivation has been during October, 1935.

Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Remarks
		Respiratory	Constitutional	
117	14th	0	+ 2 days	Sore throat, headache, malaise
118	14th	+++ 5 days	+ 2 "	Severe coryza, productive cough, red sore throat, anorexia, malaise
119	14th	+++ 5 "	+ 2 "	Nasal obstruction and discharge, productive cough, sore red edematous throat, headache, anorexia, malaise

The 14th transfer of culture strain Wh produced severe colds in 2 inoculated individuals and moderate constitutional reaction without respiratory manifestations in a 3rd.

In order to illustrate the apparently greater virulence and infectivity of the cultivated virus of common cold when transfers are made in chick embryo medium at intervals of 48 and 72 hours instead of the longer intervals of 3 to 5 to 9 days, Table I is presented. The comparison is made between cultures that have received 100 transfers or less.

Although the total number of inoculations of volunteers with the cultures transferred at 48 and 72 hour intervals is small, the increased percentage of colds produced and the increase in severity and duration of symptoms is obvious. All cultures of cold virus are now transferred routinely at 2 and 3 day intervals, 2 transfers at 48 hours and 1 at 72 hours each week.

ing codein and aspirin. Nov. 17. His symptoms remain the same as on previous day with a severe productive cough still present. Nov. 18. The symptoms are abating somewhat but he now complains of marked sore throat. Nov. 19. Feels considerably better, appetite returning. Productive cough and nasal discharge still marked, however. Discharged from isolation with rather pronounced residual manifestations. Table II shows the daily record of symptoms.

The experiments detailed in Table I indicate beyond any question that the virus of common cold can be isolated from human beings suffering from acute colds and grown *in vitro* in tissue medium containing minced 10 day old chick embryo. Cultures prepared in the manner described retain their infectivity for long periods of time and under the most favorable conditions for as many as 88 transfers in tissue medium. The dilution of the original nasopharyngeal washings serving as the source of the culture is by the 10th transfer *in vitro* already so great that any possibility of its continued presence being responsible for the experimental infection of volunteers is entirely ruled out. Intranasal inoculation of culture medium and other types of control material, although it may produce symptoms of slight irritation for short periods of time, has never caused in any individual inoculated the typical picture even of a mild common cold. The colds produced in individuals inoculated with active culture vary in severity and duration of symptoms, which in some are entirely referable to the respiratory tract and in others are combined with varying degrees of constitutional reaction. Fever has never been produced by inoculation of common cold virus and there is no significant change in the leucocyte count. Complications such as sinusitis and tracheobronchitis are occasionally observed. Blood agar plates have generally been prepared daily for the study of the nasopharyngeal flora of inoculated volunteers. No important changes in the types or numbers of the common organisms of the upper respiratory tract have been observed and the presence in the nasopharynx of such pathogenic types as pneumococcus does not seem to influence the result of inoculation of virus of the common cold.

One of the objectives of the present study has been to develop methods for the practical use of the culture virus of common cold for purposes of active immunization of chimpanzees and human beings against spontaneous colds. In view of the fact that for such an effort to be successful, living active virus would have to be used for inocula-

TABLE II

Date, *1935*	Strain Ce 126						Strain Wh 14									
	Nov. 7	8	Inoculation Nov. 9	10	11	12	13	Inoculation Nov. 14	15		16		17		18	19
									a.m.	*p.m.*	*a.m.*	*p.m.*	*a.m.*	*p.m.*		
Nasal obstruction	0	0	0	0	0	0	0	0	++	+	++	+	++	++	++	+
Sneezing	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Coryza	0	0	0	0	0	0	0	0	++	+	0	0	0	0	0	0
Nasal discharge	0	0	0	0	0	0	0	0	++	++	++	++	++	++	+	+
Cough	0	0	0	0	0	0	0	0	++	+++	+++	+++	+++		++	+
Sputum	0	0	0	0	0	0	0	0	+	+	++	++	++	++	++	+
Sore throat	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+++	+
Headache	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Anorexia	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	0
Malaise	0	0	0	0	0	0	0	0	0	0	±	+	+	+	±	0
Fever	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Throat	0	0	0	0	0	0	0	0	Slight edema		Increased redness		Marked injection		Marked injection	
Postnasal discharge	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tonsils	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

± = mild; + = moderate; ++ = marked; +++ = severe.

In the next experiment the survival of the virus under the technique of preservation employed for human vaccination was tested.

Active culture was ground up, centrifuged to remove large clumps of material, and the supernatant fluid was kept under vaseline seal at ice box temperature for 6 days. At the expiration of this time the material was inoculated into human volunteers. The results are shown in the following experiment.

Experiment 13

Volunteer No.	Material inoculated	Intensity and duration of symptoms	Remarks
107	Ro 71 preserved 6 days	0	
	Ro 75 fresh	++ 7 days	Marked coryza, cough, sore red throat, marked anorexia, malaise
108	Ro 71 preserved 6 days	0	
	Ro 75 fresh	+++ 6 days	Marked coryza, slight cough, sore red throat, headache
109	Ro 71 preserved 6 days	0	
	Ro 75 fresh	0	

The above experiment shows that storage of culture virus of common cold in the culture fluid for 6 days at ice box temperature results in complete loss of infectivity. Another transfer, No. 75, of the same strain was proven 4 days later to be active in the same volunteers when used in the fresh state. From these two experiments it is apparent that contact, even for a short period of time, with disintegrated chick embryo medium, either in the wet or dry state, completely inactivates the culture virus of common cold. For a number of practical reasons previously mentioned it has become important to be able to store the culture virus of common cold in an active state. The observation of Rivers and Ward (8) that the presence of small amounts of gum acacia exerts a protective action on the culture virus of vaccinia suggested the use of this substance for the above purpose.

To test the effectiveness of gum acacia the 88th transfer of strain Ro of cold virus was mixed with 3 per cent gum acacia, frozen and dried *in vacuo*, and stored at ice box temperature for 6 days. As a control the 88th transfer of strain Ro was preserved in the same way without the admixture of gum acacia. 3 volunteers were then inoculated, first with the virus dried without gum acacia and a few days later the same volunteers were inoculated with virus dried with gum acacia. The results are shown in the following experiment.

tions, a number of experiments have been performed which have been designed to test the viability of cold virus under different conditions of storage. In the prophylactic inoculation of human beings a minimal period of 5 days' storage is essential for the carrying out of the necessary tests of sterility of the material to be inoculated. The effect of freezing and drying was first explored.

The 18th transfer of culture strain Ro, a virus proved subsequently to be infective for human beings in the 88th transfer, was rapidly frozen, desiccated, and sealed *in vacuo*. This preparation was stored for 10 weeks at ice box temperature and then tested for activity on 3 human volunteers. Since this preparation proved inactive the same volunteers were inoculated a few days later with the original nasopharyngeal washings from which strain Ro had been cultivated and which had been dried and preserved in a similar manner for a period of 17 weeks. The results are shown in Experiment 12.

Experiment 12

Volunteer No.	Material inoculated	Intensity and duration of symptoms	Remarks
104	Culture Ro 18 dried	0	
	Original Ro virus dried	+++ 5 days	Nasal obstruction, nasal discharge, productive cough, sore throat, headache, malaise
105	Culture Ro 18 dried	0	
	Original Ro virus dried	++ 5 days	Nasal obstruction, nasal discharge, productive cough, sore throat, headache
106	Culture Ro 18 dried	0	
	Original Ro virus dried	0	

This experiment indicates that culture virus of common cold, frozen and dried under the conditions described, loses its infective power after 10 weeks storage. However, the original virus of the nasopharyngeal washings retained its activity under similar conditions for 17 weeks. It would seem, therefore, that chick embryo medium contains substances destructive to the virus, possibly disintegration products of dead tissue cells of the nature of soaps of the unsaturated fatty acid series. On the other hand, the virus of the original nasopharyngeal washings survives dried for long periods of time. It is possible that the presence of mucus in these washings exerts a protective action.

4. When kept in the original nasopharyngeal washings, the virus will survive at ice box temperature under anaerobic conditions for at least 13 days.

5. If the nasopharyngeal washings are frozen and dried *in vacuo*, the virus retains its activity for at least 4 months.

6. The virus of common cold has been proven to multiply in medium containing chick embryo tissue. Such cultures retain their capacity to produce typical infections in human beings for many transfers involving a period of several months. Attempts to cultivate the virus have been successful in seven out of eight instances.

7. Prolonged cultivation of the virus in tissue medium eventually leads to a loss of activity.

8. Strains of virus under cultivation maintain their potency best when transfers are made at 2 and 3 day intervals.

9. After removal from the incubator a culture of virus rapidly becomes inactive whether it be kept under seal in the ice box or frozen and dried *in vacuo*.

10. The destructive action of the medium can be prevented if the culture is mixed with gum acacia before freezing and drying *in vacuo*.

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Experiment 14

Volunteer No.	Material inoculated	Intensity and duration of symptoms	Remarks
110	Ro 87 without acacia	0	
	Ro 88 with acacia	+++ 7 days	Marked coryza, productive cough, sore red throat, headache
111	Ro 87 without acacia	0	
	Ro 88 with acacia	++ 6 days	Nasal obstruction and discharge, productive cough, sore red throat, headache
112	Ro 87 without acacia	0	
	Ro 88 with acacia	+++ 7 days	

This experiment shows very clearly that complete inactivation of strain Ro 87 had taken place in 5 days although the culture had been preserved in the dry state *in vacuo*; on the other hand Ro 88 similarly prepared and preserved except for the addition of 3 per cent gum acacia was highly active in the same volunteers 4 days after inoculation with Ro 87 without acacia. This result indicates that gum acacia exerts a protective action against constituents of tissue medium injurious to the cultivated virus.

From time to time during the course of this study attempts have been made to detect visible evidence of growth of the virus of common cold. None, however, has been obtained, no inclusion bodies are formed, no visible or chemical change in the media occurs which would indicate growth, nothing significant can be seen with the dark field microscope; nor do colonies form either aerobically or anaerobically on solid media.

SUMMARY AND CONCLUSIONS

1. Studies of the cultivation of the virus of common cold in tissue medium, and the capacity of the culture virus to induce infection in human volunteers are reported.

2. Detailed descriptions are given of the methods employed to isolate the virus, preserve and cultivate it, and to test its activity in human volunteers.

3. The virus of common cold can easily be isolated from properly selected patients and cultivated in tissue medium.

STUDIES ON THE VIRUS OF INFLUENZA

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From the time of its discovery in 1891 until 1918 the bacillus of Pfeiffer was generally regarded as the cause of influenza; as a consequence of this belief nearly all of the huge volume of investigation done at the time of the recent great pandemic concerned itself with this organism. These studies gave rise to many confused and contradictory results, and ended by casting doubt upon the etiological significance of Pfeiffer's bacillus, or any other of the visible microorganisms that could be cultivated from the respiratory tract. As a result of this uncertainty a filtrable virus was sought in connection with influenza, and as early as August, 1918, Selter (1) reported the production of a mild influenza-like disorder in one of 2 volunteers who inhaled the filtered throat secretions from an active case of the disease. A few other reports (2–4) seemed to confirm this observation, but the very small number of experiments, the lack of quarantine procedures, variation in incubation period, and the fact that an epidemic was in progress at the time, surrounded these early essays with an atmosphere of doubt. In addition, it is noteworthy that other observers (5–7) failed to obtain like results in a larger series of attempts, and by 1922 it is safe to say that the evidence against a filtrable virus as the cause of influenza was more convincing than that in favor of it.

Since 1918 there has been no great pandemic of influenza; the existence, however, of so called interpandemic influenza, ordinarily occurring in winter in rather localized outbreaks, in which many of the cases closely resemble the pandemic variety, has tempted investigators in recent years to direct their attention again to this disease. In 1931 Long and his coworkers (8) reported the appearance in chimpanzees of a febrile disease after inoculation with filtered secretions from influenza patients; in 1932, however, Costa-Mandry *et al.* (9) failed to achieve the same result in human volunteers with material obtained during the Puerto Rico epidemic of that year.

Our own studies of the common cold soon led us to the belief that this problem could not be separated from that of influenza. Clinical and epidemiological studies alike suggest that many examples of influenza during a typical outbreak so closely resemble the common cold as to be indistinguishable from it. This has been observed in isolated communities in the far north when infection has been introduced from the outside by a single individual. A study and knowledge of both diseases is therefore essential to the determination of the value of any form

Chart 1

Volunteer 25. Virus: filtered washing from M.S.

Date, 1931	Observation period			Inoculation Jan. 19	20	21	22
	Jan. 16	17	18				
Nasal obstruction	0	0	0	0	+	0	+
Sneezing	0	0	0	0	+	+	0
Nasal discharge	0	0	0	0	+	0	±
Cough	0	0	0	0	+	0	+++
Sore throat	0	0	0	0	0	0	0
Headache	0	0	0	0	+	0	0
Malaise	0	0	0	0	0	0	0
Fever	0	0	0	0	0	0	0

± very mild, + mild, ++ moderate, +++ marked, ++++ severe.

As will be seen from the foregoing chart, this subject developed very mild symptoms following intranasal inoculation; these were entirely referable to the upper respiratory passages, with the exception that cough became marked on the day of discharge from the hospital. Constitutional symptoms were absent. The type of response manifested by this individual indicated the presence in the filtrate of a virus similar in action to the virus of the common cold.

4 days after discharge he reappeared at the hospital with a temperature of 103° *per os*, complaining of cough, headache, and marked prostration. He stated that his cold became worse immediately after discharge, and that he felt feverish the next day. He was found to have bronchitis and sinusitis, and remained 2 weeks under treatment on the medical wards. The significance of the relapse and intensification of symptoms is not clear. Perhaps outside exposure caused a complication of his infection.

Volunteer 26, who was simultaneously inoculated with the same material, manifested no signs of infection.

On the basis of this single experiment tentative opinion only could be formed that there existed in one patient suffering from interpandemic influenza, a virus similar in action in an experimental individual to the virus of common cold.

2. *Experiments with Virus of Interpandemic Influenza Cultivated in Vitro.*—Strain F was derived from the following case.

In February, 1932, a telephone operator was abruptly seized with nausea, followed by headache, backache, and a shaking chill. Temperature 1 day later was 103°, W.B.C. 7,000. She showed slight cyanosis, injected conjunctivae,

of prophylactic immunization. In an accompanying paper (10) we have reported in detail our investigations of the common cold which convinced us that the primary etiological agent of this disease is a filtrable virus. Having developed a technique for the study of this virus, it has seemed advisable to apply this same technique to the study of interpandemic influenza. In 1933 we reported (11) the cultivation *in vitro* of a virus derived from a patient with influenza, and the cultivation of a second strain was noted in 1934 (12).

In 1933 (13) Smith, Andrewes, and Laidlaw announced that they had succeeded in initiating a virus disease of ferrets, transmissible in series, with filtered throat secretions of influenza patients, and later on (14) observed that the influenza virus after passage through ferrets became also pathogenic for mice. Similar observations have been made by Francis (15). Although it has not yet been possible to produce influenza in human beings with this virus, nevertheless the isolation of a number of strains from different parts of the world and their serological identity as shown by both Andrewes (16) and Francis (17), and the study of the protective power of convalescent serum by the latter, strongly suggest that these workers are dealing with the virus of interpandemic influenza. Recently Francis (18) has announced that the virus obtained from mouse lung can be cultivated in tissue medium under aerobic conditions.

The object of the present paper is to report in detail the experiments we have performed with the virus of influenza from 1931 to 1935.

Methods

The methods used in isolating the virus, preserving it, cultivating it, and testing for its activity in human volunteers, have been described in complete detail in a previous publication (10).

RESULTS

1. Attempted Transfer of Influenza from Individual to Individual.—The first experiment was performed in January, 1931, and consisted of the intranasal inoculation of a fresh, bacteria-free filtrate of nose and throat washings from a patient with influenza.

Case.—M. S., a student nurse free from upper respiratory symptoms for a period of 2 months, suddenly noted cough, malaise, and moderate sore throat; later on she developed nasal obstruction and discharge. Temperature at onset 101.6° *per os*, W.B.C. 7,300. She remained in bed 4 days, and returned to duty on the 6th day. During this month there was a rather high incidence of influenza in New York City. The washing was taken 24 hours after the onset of the disease.

This experiment indicates that from a typical example of the interpandemic form of influenza a filtrable virus was cultivated in tissue medium. This cultivated virus when inoculated intranasally into 2 human volunteers produced in both volunteers the symptoms of a severe common cold. The degree of constitutional reaction was slight and neither of the volunteers experienced a rise in temperature. There was no characteristic alteration in the leucocyte count. In view of the similarity of the symptomatology to that of common cold, an effort was made to determine if infection with the above culture virus gave a cross immunization against the culture virus of common cold. An attempt was first made to test the immunity of the 2 above individuals against infection with the homologous culture virus strain F.

The men were allowed to go home and readmitted to the isolation quarters after the lapse of 3 weeks when they were again inoculated intranasally with strain F transfer 26. The results are shown in the following chart.

Chart 4

Volunteer 55 b. Virus F 26								
Date, 1932	May 20	Inoculation May 23	24	25	26	27	28	
Nasal obstruction	0	0	+++	+	++	++	0	
Sneezing	0	0	±	0	0	+	0	
Nasal discharge	0	0	+	++	0	++	0	
Cough	0	0	+	±	0	+	0	
Sore throat	0	0	0	0	0	0	0	
Headache	0	0	0	0	±	0	0	
Malaise	0	0	0	0	0	0	0	
Fever	0	0	0	0	0	0	0	

Volunteer 56 b. Virus F 26								
Date, 1932	May 20	23	24	25	26	27	28	
Nasal obstruction	0	0	++	+	+++	++	±	
Sneezing	0	0	+	+	0	±	0	
Nasal discharge	0	0	++	+	0	++	±	
Cough	0	0	+	++	0	0	0	
Sore throat	0	0	0	0	0	0	0	
Headache	0	0	+	++	++++	++	0	
Malaise	0	0	0	0	0	0	0	
Fever	0	0	0	0	0	0	0	

some nasal obstruction, and a dry cough. Fever lasted 4 days, and on the 3rd day the white blood count was 4,800. Influenza was prevalent at the time. This patient had also a very mild chronic cystitis which was not altered by the superimposed respiratory infection.

A tissue culture was initiated with material derived from this patient in the manner already described (10). Transfers were made at 3 to 5 day intervals. In the 19th transfer of the culture, Strain F was tested on 2 volunteers.

Chart 2

Volunteer 55 a. Virus F 19

Date, *1932*	Apr. 29	Inoculation May 2	3	4	5	6	7	8	9
Nasal obstruction	0	0	++	++	+	++	+++	++	±
Sneezing	0	0	+	0	+	0	+++	+	0
Nasal discharge	0	0	++	++	++	+	+++	+	+
Cough	0	0	+	++	+	++	++	±	0
Sore throat	0	0	0	+	0	0	0	0	0
Headache	0	0	+	0	±	0	±	0	0
Malaise	0	0	+	+	0	0	0	0	0
Fever	0	0	0	0	0	0	0	0	0

This patient, as is seen from the above chart, developed a typical head cold of more than average severity. There was an exacerbation of symptoms on the 5th day, but throughout its course the disease had obvious characteristics of a head cold, with mild constitutional reaction and no fever. On the 3rd day the leucocytes were 9,500.

Chart 3

Volunteer 56 a. Virus F 19

Date, *1932*	Apr. 29	Inoculation May 2	3	4	5	6	7	8	9
Nasal obstruction	0	0	+++	+++	++	++	+	+	±
Sneezing	0	0	++	+	+	0	0	0	0
Nasal discharge	0	0	++++	++++	++	++	+	±	±
Cough	0	0	++++	++++	+	0	±	+	±
Sore throat	0	0	±	+	+	±	0	±	±
Headache	0	0	++++	++++	++	0	0	0	0
Malaise	0	0	+	+	+	+	0	0	0
Fever	0	0	0	0	0	0	0	0	0

Like the preceding volunteer, this man developed symptoms characteristic of a severe head cold. There was only a mild degree of malaise, and no fever. On the 3rd day the leucocytes were 10,050.

The history of culture strain F as depicted in Charts 3, 4, and 5 presents some interesting facts.

The strain of virus was isolated from a patient suffering from a typical attack of influenza of the interpandemic form. There was a moderately high incidence of similar clinical types of influenza at the time. The first series of volunteers responded to inoculation with the symptoms of a severe common cold with marked upper respiratory manifestations and slight or no constitutional reaction. This was followed by a period during which inoculation of the culture produced little or no symptoms of any kind. During this period a strain of the culture was transferred to London where a number of experimental inoculations were made, the majority of which gave no positive evidence of infection. In the series of volunteers 64 to 70 the symptomatology of the experimental disease following inoculation changed markedly. These individuals, after an incubation of 24 hours or longer, all developed rather marked constitutional symptoms consisting of generalized aches and pains, lethargy, and anorexia. In addition to these manifestations they suffered from a mild degree of fever, which up to this time had never been observed in any of the experimentally produced upper respiratory infections. On the other hand the symptoms of irritation of the respiratory tract were very slight. 9 months after these inoculations the culture had entirely lost its capacity to induce symptoms in an inoculated individual.

In January, 1933, a second strain of influenza virus, strain T, was cultivated.

A graduate nurse, free from respiratory symptoms for the previous 3 months, suddenly noted general slight malaise, a sensation of swelling in the throat, and some cough. In the evening the malaise deepened, and she had chilly sensations, pain in the back and neck, and prostration. At the end of a restless night her temperature was 101.6°F., W.B.C. 5,800. Aside from some swelling of the lymphoid tissue in the pharynx, there were no significant physical signs. At the end of 48 hours the fever had subsided, and the patient made a rapid recovery. Influenza was prevalent at this time.

A tissue culture was initiated with material from this patient in the usual manner, transfers being made at 3 to 4 day intervals. The strain was first tested on 2 volunteers in the 18th transfer. In this particular experiment an inoculation of filtered nasopharyngeal washings from a normal individual was made 3 days before the actual test with strain T. The object of this inoculation was to ascertain if a virus capable of inducing respiratory symptoms might be carried by a normal person during the winter months. This inoculation was followed by no signs of infection. The results of inoculation with strain T are given in Chart 6.

Chart 6 shows that both men developed head colds, one somewhat more severe than the other. Both had an exacerbation of symptoms on the 4th day. Symptoms were, for the most part, limited to irritation of the upper respiratory tract, and the degree of general malaise was insignificant. Neither man had fever.

The above chart shows that both volunteers were susceptible to reinoculation with the F strain of influenza virus 3 weeks after their original infection. In both the disease produced was a moderate head cold without constitutional reaction. Both men declared that the symptoms were quite similar to those produced by the first inoculation, although less than half as severe, an observation borne out by a comparison of the two charts.

This experiment demonstrated that an infection with a strain of culture virus did not confer a solid immunity against reinfection with the same strain. Since little immunity appears under the circumstances of the experiment to develop against the homologous strain, the method is not suitable for cross immunity tests.

Culture strain F was subsequently carried *in vitro* until November, 1933. The total period of cultivation from the time of isolation was approximately 20 months. The intervals of transfer varied from 3 to 5 days. During this period human volunteers were inoculated from time to time and the results of these inoculations are presented in summary in Chart 5.

Chart 5

Date	Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Fever
			Respiratory	Constitutional	
1932			*days*	*days*	*°F., p.o.*
July	59	37	+2	0	0
"	60	37	0	0	0
Oct.	61	58	±2	±2	0
"	62	58	±2	0	0
"	63	58	±2	±1	0
Nov.	64	65	0	++2	99.2
"	65	65	0	++3	99.0
"	66	65	+5	++2	99.0
1933					
Jan.	67	80	±5	++3	99.4
"	68	80	+5	+4	99.4
"	69	88	+4	+++4	99.0
"	70	88	±3	+3	99.0
Oct.	77	156	0	0	0
"	78	156	0	0	0
"	79	156	0	0	0
Nov.	83	162	0	0	0
"	84	162	0	0	0

Chart 7—Concluded

Volunteer 82. Virus T 80

Date, 1933	Oct. 27	Inoculation Oct. 30	31	Nov. 1	2	3	4
Nasal obstruction	0	0	±	++	++	++	+
Sneezing	0	0	0	0	0	0	0
Nasal discharge	0	0	++	++	+	+	+
Cough	0	0	+	++	++	+++	+
Sore throat	0	0	0	+	0	+	0
Headache	0	0	0	0	0	0	0
Malaise	0	0	±	+	0	0	0
Fever	0	0	0	0	0	0	0

Volunteer 81. Virus T 80

Date, 1933	Oct. 27	30	31	Nov. 1	2	3	4
Nasal obstruction	0	0	++	+++	+++	+	+
Sneezing	0	0	+	+	0	0	0
Nasal discharge	0	0	++	++	++	+	+
Cough	0	0	+	+++	+++	++	+
Sore throat	0	0	0	0	0	0	0
Headache	0	0	0	++	+	0	0
Malaise	0	0	+	+	+	+	0
Fever	0	0	0	0	0	0	0

These results show that strain T was still capable of producing infection in the 80th transfer, the symptoms which it produced being chiefly those of upper respiratory irritation. Volunteer 81 showed a

Chart 8

Volunteer 88. Virus T 113 and 114 mixed

Date, 1934	Feb. 23	Inoculation Feb. 26	27	28	Mar. 1	2	3
Nasal obstruction	0	0	+	++	+	++	0
Sneezing	0	0	±	±	0	0	0
Nasal discharge	0	0	+	±	+	+	0
Cough	0	0	+	+	0	0	0
Sore throat	0	0	0	0	±	0	0
Headache	0	0	0	++	±	±	0
Malaise	0	0	0	0	±	0	0
Fever	0	0	0	0	0	0	0

Chart 6

Volunteer 73. Virus T 18

Date, *1933*	Mar. 1	Inoculation Mar. 6	7	8	9	10	11	12	13
Nasal obstruction	0	0	+	±	0	++	±	0	0
Sneezing	0	0	±	0	±	0	0	0	0
Nasal discharge	0	0	+	++	+	+++	+	+	+
Cough	0	0	0	±	±	++	+	±	0
Sore throat	0	0	0	0	0	0	0	0	0
Headache	0	0	++	+	±	±	0	0	0
Malaise	0	0	0	+	0	0	0	0	0
Fever	0	0	0	0	0	0	0	0	0

Volunteer 74. Virus T 18

Date, *1933*	Mar. 1	6	7	8	9	10	11	12	13
Nasal obstruction	0	0	0	±	±	+	+	±	±
Sneezing	0	0	±	++	+	+	+	±	0
Nasal discharge	0	0	+	++	++	++	++	++	++
Cough	0	0	0	+++	++	+++	+	±	+
Sore throat	0	0	0	0	0	+	0	0	0
Headache	0	0	±	±	±	±	0	0	0
Malaise	0	0	±	0	±	0	0	0	0
Fever	0	0	0	0	0	0	0	0	0

Strain T was next tested in the 80th transfer; the results are given in Chart 7.

Chart 7

Volunteer 80. Virus T 80

Date, *1933*	Oct. 27	Inoculation Oct. 30	31	Nov. 1	2	3	4
Nasal obstruction	0	0	+	±	0	0	0
Sneezing	0	0	++	0	0	0	0
Nasal discharge	0	0	++	++	0	0	0
Cough	0	0	±	+	++	+	0
Sore throat	0	0	0	0	0	0	0
Headache	0	0	0	+	0	0	0
Malaise	0	0	++	0	0	0	0
Fever	0	0	0	0	0	0	0

Chart 9

Volunteer 93. Virus R 18

Date, 1934	May 25	Inoculation May 28	29	30	31	June 1	2	3	4
Nasal obstruction.	0	0	++	+	0	0	0	0	0
Sneezing.	0	0	++	±	0	0	0	0	0
Nasal discharge...	0	0	++	++	+	0	0	0	0
Cough.	0	0	++	+	+	0	0	0	0
Sore throat.	0	0	+	+	0	0	0	0	0
Headache.	0	0	+	+	0	0	0	0	0
Malaise.	0	0	+	0	0	0	0	0	0
Anorexia.	0	0	0	0	0	0	0	0	0
Vomiting.	0	0	0	0	0	0	0	0	0
Prostration.	0	0	0	0	0	0	0	0	0
Dizziness.	0	0	0	0	0	0	0	0	0
Fever.	0	0	0	0	0	0	0	0	0

Volunteer 94. Virus R 18

Date, 1934	May 25	28	29	30	31	June 1	2	3	4
Nasal obstruction.	0	0	±	+	+	++	+	0	0
Sneezing.	0	0	0	0	0	+	0	0	0
Nasal discharge...	0	0	++	++	++	++	+	0	0
Cough.	0	0	±	+	++	+	±	0	0
Sore throat.	0	0	0	0	0	0	0	0	0
Headache.	0	0	±	+	+	+	±	0	0
Malaise.	0	0	++	+++	+	++	0	+	0
Anorexia.	0	0	0	+	±	±	0	0	0
Vomiting.	0	0	0	+	0	0	0	0	0
Prostration.	0	0	0	+	0	0	0	0	0
Dizziness.	0	0	+	+	0	0	0	0	0
Fever.	0	0	0	0	+	0	0	0	0

Volunteer 95. Virus R 18

Date, 1934	May 25	28	29	30	31	June 1	2	3	4
Nasal obstruction.	0	0	+++	++	±	±	+	0	0
Sneezing.	0	0	+	0	0	0	0	0	0
Nasal discharge...	0	0	+++	++	+	+	+	+	0
Cough.	0	0	+	++++	+++	+++	++	±	+
Sore throat.	0	0	0	+	++	0	±	0	0
Headache.	0	0	+++	+	++	+++	±	±	±
Malaise.	0	0	+++	+++	++	+++	±	±	0
Anorexia.	0	0	+	++	+	+++	±	0	0
Vomiting.	0	0	0	+++	Nausea	+	0	0	0
Prostration.	0	0	0	++	+	0	0	0	Weakness
Dizziness.	0	0	+	+	+	0	0	+	+
Fever.	0	0	0	101° F.C.	0	0	0	0	0

moderate degree of malaise. No elevation of temperature occurred in this group.

Strain T was tested for the last time on one volunteer who was inoculated with a mixture of the 113th and 114th transfers of the culture. The result is shown in Chart 8.

Strain T, therefore, showed some infective power after more than 100 transfers of the culture. No subsequent tests were made. To summarize our experience with this strain of virus derived originally from a mild case of influenza, strain T produced in the 6 volunteers inoculated symptoms for the most part referable to infection of the upper respiratory tract and of the type observed in common cold. The degree of constitutional reaction was slight and there was no fever.

In March, 1934, a fourth strain of influenza virus, strain R, was cultivated from a patient suffering from influenza which occurred at that time as a local epidemic in the Pelham Home.

This establishment houses a population of 35 girls, all of whom are rheumatic fever subjects. In March, 1934, a serious epidemic of influenza took place in the Home, apparently introduced by the cook. Coburn and Pauli (19) have reported the clinical aspects of this outbreak in detail, together with a bacteriological and immunological study of the individual cases. The main clinical features of the disease at the Pelham Home were: rather sudden onset, fever of about 104° for 3 days, with marked prostration, nausea, vomiting, and insignificant symptoms of upper respiratory irritation. As the epidemic progressed a variation in the type of response took place, so that the intermediate cases showed increasing symptoms of respiratory irritation and decreasing fever. The last cases of the disease were afebrile and resembled the common cold. Leucopenia was not observed during the outbreak. Strain R was derived from a typical example of the disease at the beginning of the epidemic. It was cultivated in tissue medium for 18 transfers and then tested in 3 volunteers. The results are given in Chart 9.

These protocols show that the 18th transfer of strain R of influenza virus produced quite different effects in 3 volunteers inoculated with the same dosage under identical conditions.

Volunteer 93 experienced the symptoms of a common cold of moderate intensity which lasted 3 days. No. 94 had symptoms of a severe cold lasting 5 days, and in addition, on the first 4 days there was a marked constitutional reaction consisting of prostration, dizziness, loss of appetite, and pain in the neck. On the night of May 29 he vomited several times. There was no fever.

were cultivated from two examples of what appeared to be the sporadic type of influenza.

Strain Ra was obtained from a 41 year old man with a past history of maxillary sinusitis. 4 days before admission he caught cold and felt feverish; he had nasal discharge, dry cough, and increasing malaise. The day before admission his temperature was said to have been 104°. On admission it was 101°, and became normal 2 days later. W.B.C. 5,900. X-ray examination was suggestive of sinusitis, and 6 days after admission the right antrum was irrigated and an improvement of the patient's condition resulted.

A nasopharyngeal washing was obtained on the day of admission, and a culture initiated in the usual manner. The final diagnosis of this patient's condition was acute sinusitis.

Strain To was obtained from a patient with the following history: A 45 year old American housewife, after exposure at the house of a friend to a case of what was called influenzal pneumonia, suddenly noticed chilly sensations, and extreme prostration. Her temperature was found to be 103°F. The next day she was admitted to the hospital. There were no symptoms of respiratory irritation. Her only complaints were feverishness, great prostration, and generalized aching pains. Physical examination showed an acutely ill woman with some heliotrope cyanosis and a slightly red throat. W.B.C. 3,250. Fever lasted 3 days, the highest temperature being 104°. W.B.C. remained below 7,000 for 2 weeks and the patient remained in the hospital 27 days on account of extreme asthenia which disappeared very slowly. During most of this period the blood pressure was low, and for the first week of her hospital stay capillary fragility was increased as was indicated by a positive tourniquet test.

Although no epidemic was in progress at the time, the symptoms from which this individual suffered exactly corresponded to those of uncomplicated influenza of the pandemic variety. A nasopharyngeal washing was obtained 48 hours after the onset of the disease and cultivated in tissue medium for 13 transfers before inoculation of human volunteers. The volunteers used in the test of culture To had a few days previously been inoculated with culture Strain Ra, which had proven inactive. The results of inoculation with both Strain Ra and To are presented in Chart 11.

These protocols show that inoculation of strain Ra produced no infection in either of the 2 volunteers. However, both were found to be susceptible a few days later to infection with strain To. In view of the fact that the final diagnosis of patient Ra's condition was acute sinusitis, it is possible that no filtrable virus was present in the respiratory passages.

The type of infection produced by inoculation of strain To was different in the 2 volunteers tested. In volunteer 120, constitutional symptoms—pains about

The effect of inoculation in volunteer 95 was striking; while coryzal symptoms were not prominent after the first day, severe constitutional manifestations developed which did not disappear entirely for 6 days, and a cough which was still present on discharge from the hospital a week after inoculation.

On the 2nd day of his disease he presented an appearance like that of influenza; he was prostrated and complained of aching pains, chilly sensations and sweating, marked dizziness, nausea, and vomiting. His eyes were suffused, face flushed, throat diffusely red. Lungs were clear. The mouth temperature was 101°F. Leucocytes were 10,900. Except for the fact that his fever lasted only a day, the clinical picture was like that seen in interpandemic influenza of average severity. The volunteer declared that the experimental disease was very similar to, though somewhat less severe than, an attack of influenza he had experienced in 1918 while in the army.

Strain R was tested for activity in 5 volunteers in later transfers of the culture; the results of these tests are briefly summarized in Chart 10.

Chart 10

Date	Volunteer No.	Transfer of culture	Intensity and duration of symptoms		Fever
			Respiratory	Constitutional	
1934					
Sept.	96	R 53	0	0	0
"	97	R 53	0	0	0
"	98	R 63	+3 days	0	0
Oct.	99	R 63	±2 "	0	0
"	100	R 63	+3 "	0	0

Chart 10 shows that of 5 inoculations into human volunteers of later transfers of the culture strain R a mild coryza resulted in 3 instances.

In summary, strain R was isolated from a patient during an outbreak of influenza which occurred in a small semi-isolated population. When tested in the 18th transfer this culture virus gave rise to one infection resembling clinical influenza, to one "grippy" cold, and to one coryzal type of cold. Subsequent inoculations of the 53rd and 63rd transfers resulted in mild colds of the coryzal type in which there were no constitutional symptoms.

In October and November, 1935, two additional strains, Ra and To,

ing Pneumococcus Types I, II, or III, or *S. hemolyticus* were not used for experimental infection. However, certain individuals throughout the period of an experiment harbored in their throats pneumococci of types other than those mentioned and frequently considerable numbers of *H. influenzae*. The presence of such organisms during the course of an experimental infection did not seem, as far as could be determined by observation, to alter the severity or character of the clinical manifestations when compared with the experimental disease in individuals free from pneumococci or *H. influenzae*. We have previously observed that during a spontaneous or experimental cold in chimpanzees (20), when *H. influenzae* is present in the nasopharynx of these animals a shift from the R form of this organism to the S form may take place. No such change of type of *H. influenzae*, though carefully looked for, was observed in human beings. In a number of instances the virulence of the pneumococcus present was tested by mouse inoculation both before and after inoculation. No change in virulence was observed. It is true, however, that complications such as purulent sinusitis and severe bronchitis were not observed and it is possible that the conditions under which the experiments were performed diminished the liability to such complications.

DISCUSSION

In this paper are presented the results of one attempt to transfer influenza from one human being to another by direct inoculation of a bacteria-free filtrate of nasopharyngeal washings and of five attempts to cultivate in tissue medium a filtrable virus from human cases of influenza both of the epidemic and sporadic type. As a consequence of this study the conclusion can be drawn that there is present in filtered nasopharyngeal washings derived from an acute case of influenza a non-bacterial filtrable agent which is capable of producing in human volunteers infection of the upper respiratory tract and that this agent can be successfully propagated in a medium containing minced chicken embryo. The cultivated agent produces no visible change in the medium in which it grows nor can visible bacterial forms be demonstrated by subculture or by microscopic study. It belongs to the class of filtrable viruses.

In all, 39 human volunteers have been experimentally inoculated

Chart 11

Volunteer 120

Date, *1935*.....	Virus Ra 8 and 9						Virus To 13						
	Nov. 23	24	Inoculation Nov. 25	26	27	28	Inoculation Nov. 29	30	Dec. 1	2	3	4	5
Nasal obstruction..	0	0	0	0	0	0	0	0	++	++	++	+++	+
Sneezing....	0	0	0	0	0	0	0	0	0	0	0	0	0
Nasal discharge....	0	0	0	0	0	0	0	0	+	++	++	++	+
Cough.....	0	0	0	0	0	0	0	0	+++	+++	++	++	±
Sore throat..	0	0	0	0	0	0	0	0	0	0	0	0	0
Headache...	0	0	0	0	0	0	0	0	++++	+	+	++	±
Malaise.....	0	0	0	0	0	0	0	+	++	++	++	±	0
Fever.....	0	0	0	99.4°	0	0	0	0	0	0	0	0	0

Volunteer 121

Date, *1935*.....	Virus Ra 8 and 9						Virus To 13						
	Nov. 23	24	25	26	27	28	29	30	Dec. 1	2	3	4	5
Nasal obstruction..	0	0	0	0	0	0	0	+	+	+	+	+	±
Sneezing....	0	0	0	0	0	0	0	0	0	0	0	0	0
Nasal discharge....	0	0	0	0	0	0	0	++	++	++	+	+	±
Cough.....	0	0	0	0	0	0	0	++	+++	+++	++	±	±
Sore throat..	0	0	0	0	0	0	0	+	+++	+	+	0	0
Headache...	0	0	0	0	0	0	0	0	0	0	0	0	0
Malaise.....	0	0	0	0	0	0	0	0	+	±	0	0	0
Fever.....	0	0	0	0	0	0	0	0	0	0	0	0	0

the head and ears, dizziness, insomnia, and anorexia—were prominent features of the experimental disease. In addition, symptoms of upper respiratory irritation and severe cough were present. There was no fever. In volunteer 121 the symptoms resulting from inoculation were of the coryzal type accompanied by moderately severe cough and sore throat, and there was little or no constitutional reaction.

Throughout the course of these studies careful observation of the bacterial flora of the nasopharynx of inoculated volunteers was maintained. In accordance with our usual procedure individuals harbor-

case histories of the patients from which these three were derived indicates that they approached more closely the epidemic type of influenza than did the two (strain T and the filtrate experiment) in which the virus obtained was indistinguishable from the common cold virus. In only one (volunteer 121) of 39 volunteers inoculated has the clinical picture of human influenza been reproduced. This was effected by the virus (R) from the Pelham Home outbreak—an outbreak which aside from its purely local character conformed to the epidemic pattern. It is interesting that of 2 additional volunteers inoculated at the same time and with the same material and maintained under identical conditions, one developed a mild coryzal type of common cold and the other a cold of the so called grippy variety.

Lastly, one attempt was made to cultivate a virus from a case of upper respiratory disease with high fever which appeared to be acute sinusitis rather than influenza. No virus was obtained from this case in an attempt made 4 days after the onset of symptoms.

SUMMARY AND CONCLUSIONS

1. Evidence is presented indicating the presence of a filtrable virus in the nasopharyngeal secretions of individuals suffering from influenza.

2. An attempt to transfer influenza from one human being to another by means of filtered nasopharyngeal washings resulted in the production in the inoculated volunteer of a common cold.

3. A filtrable agent has been cultivated in tissue medium from the filtered nasopharyngeal washings of patients with influenza.

4. Inoculation of the cultivated virus into human volunteers results for the most part in the production of a severe common cold with a tendency to pronounced constitutional reaction.

5. In one instance following inoculation of culture virus an infection clinically resembling influenza has been produced.

6. The more closely the source of the virus approached the type of epidemic influenza, the more likely the virus was to provoke constitutional symptoms.

7. The presence of certain pathogenic bacteria in the upper respiratory tract of inoculated individuals was not observed to modify the course or character of the experimental infection.

with this filtrable agent. The outstanding outcome of these inoculations has been that in every instance but one the inoculation, when positive, whether of fresh filtered washings or of the cultivated virus, has resulted in the production of an infection more nearly resembling the common cold than human influenza. A significant explanation of these results cannot be given at the present time. It is, of course, well known that a large number of upper respiratory infections occurring during a typical epidemic of influenza resemble in character the common cold. Furthermore, in outbreaks of respiratory infection in colonies in the far north which originate from a single source, types of infection resembling both influenza and common cold are present at the same time.

Other explanations of the predominant occurrence of infections resembling common cold in the experimentally inoculated individuals may be offered. It is not unlikely that the conditions of cultivation of the virus result in a diminution of virulence so that the infection following inoculation manifests symptoms of less severity. Moreover, the importance of virulent secondary organisms in the production of the typical picture of human influenza is not understood at the present time, and the absence of such organisms from the nasopharynx of the inoculated volunteers may have influenced the character of the symptomatology. In addition, the fact that all volunteers during the period of infection were maintained in the favorable and protected environment of a private hospital suite may also have influenced the character of the experimental disease. Finally, there is no way at the present time of differentiating conclusively the virus of common cold from that of influenza. That these two viruses are closely related in their manifest activity is extremely likely and that they are close to one another in biological nature seems probable.

On the whole, experimental infections induced in human beings by inoculation of the filtrable virus cultivated from influenza have been more severe in their symptomatology than a comparable series of infections arising from inoculation of the cultivated virus of the common cold. Furthermore, the tendency to exhibit a more pronounced constitutional reaction after inoculation of influenza virus is more conspicuous. These phenomena appear in connection with three of the viruses studied—designated strains F, R, and To. A study of the

RELATION OF THE HYPOPHYSIS TO THE SPLEEN

I. Effect of Hypophysectomy on Growth and Regeneration of Spleen Tissue

II. The Presence of a Spleen-Stimulating Factor in Extracts of Anterior Hypophysis

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Plates 43 to 45

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Incidentally to other studies, Smith (1) noted that removal of the hypophysis in rats is followed by a decrease in size of the spleen as well as of other viscera and endocrine organs. Daily homeotransplants of anterior hypophysis caused return of the viscera to normal or nearly normal proportions. In a series of immature hypophysectomized dogs, Houssay and Lascano-Gonzalez (2) observed that the arrest of growth of the animals was accompanied by a parallel cessation of growth of the spleen. The number of Malpighian corpuscles was more numerous in the hypophysectomized than in the control animals, and these were larger in size. In dogs hypophysectomized after the age of 10 weeks, the follicles of the spleen were more numerous but not larger than in the controls. The rhythm of splenic contraction diminished in the hypophysectomized dog. They concluded, however, that there is no atrophy of the spleen in this animal following removal of the hypophysis.

The studies reported in the present communication were undertaken to learn more of the relationship that exists between the activity of the anterior hypophysis and the structure and function of the spleen.

The effect of complete and partial hypophysectomy on the size of the spleen in the rat was determined in a large series of mature albino rats at various intervals after operation. The results of daily injec-

8. On prolonged cultivation the virus loses the capacity to infect human volunteers.

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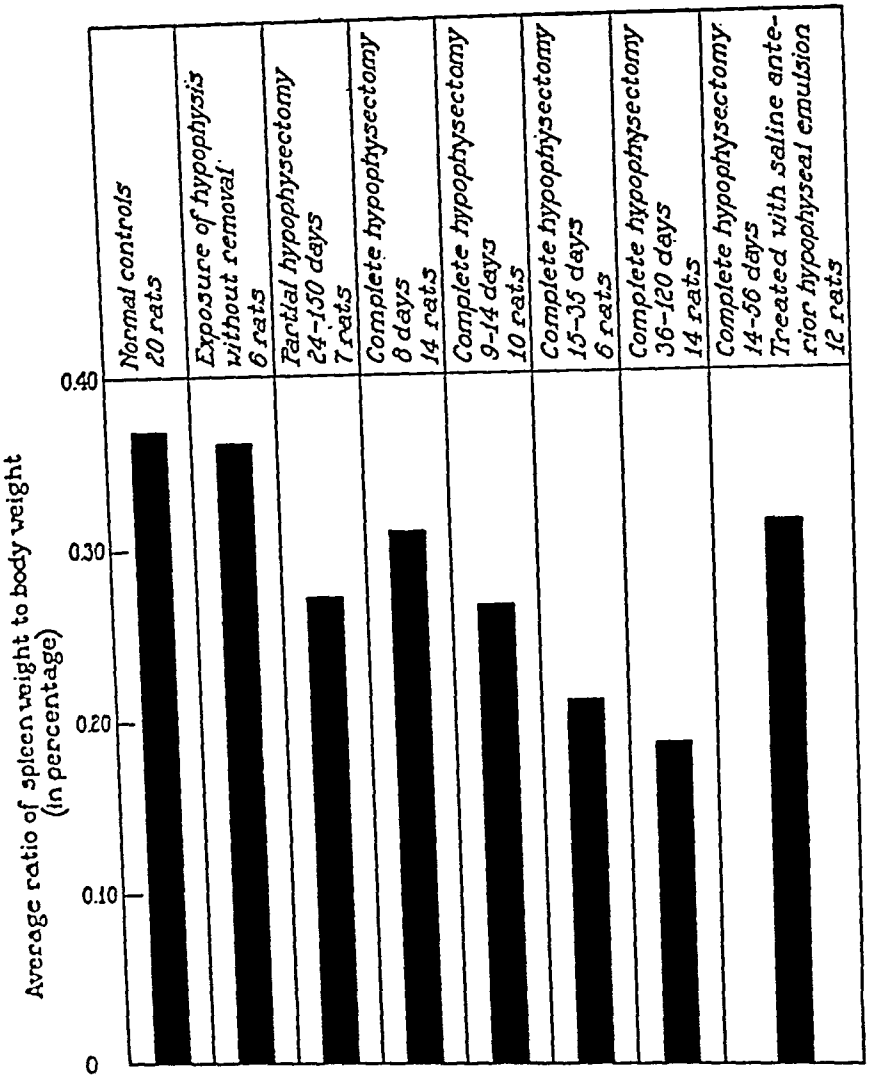


TEXT-FIG. 1. The effect of partial and complete hypophysectomy in adult rats on the relative weight of the spleen. The height of the bars indicates the average ratios of spleen weight to body weight expressed in hundredths of 1 per cent.

In the series of hypophysectomized rats, no rat was included that presented evidence of infection at autopsy or had received any treatment or injection other than indicated. In each animal the weights of the internal organs, the weight of

tions of emulsion of fresh anterior hypophysis of cattle on the size of the spleen of hypophysectomized rats was observed. In a second group of experiments the effect of hypophysectomy on the regenerative capacity of the splenic stump after removal of four-fifths of the spleen was studied. In a third group of experiments the effect of repeated injections of emulsions of fresh anterior lobe, and of various fractions of the anterior lobe on the growth of the spleen in normal adult albino rats was determined.

TABLE I

The Effect of Hypophysectomy on the Size of the Spleen

No. of rats	Procedure	Interval between operation and death	Ratio of spleen weight to body weight			
			Average	Mean	Maximum	Minimum
		days				
20	Normal		0.369	0.375	0.60	0.21
6	Exposure of pituitary (operation control)	30–120	0.361	0.40	0.426	0.25
7	Partial* hypophysectomy	24–150	0.273	0.284	0.49	0.14
14	Complete hypophysectomy	8	0.31	0.28	0.55	0.14
10	" "	9–14	0.268	0.275	0.42	0.13
6	" "	15–35	0.213	0.24	0.40	0.18
14	" "	36–120	0.189	0.19	0.32	0.12
11	" "	14–30	0.33	0.29	0.74	0.20
	Treated with emulsion					

* The results in this group were variable (see text for explanation).

The Effect of Hypophysectomy on the Size of the Spleen

The rats used in these experiments were all of uniform stock, raised in our laboratory during a period of 12 years. Though of original Wistar strain they were carriers of *Bartonella muris*. In previous work it had been observed that hypophysectomy does not affect this latent infection. All the rats were between 3 and 4 months of age at the onset of the experiment. In the first group of experiments, a total of 89 rats were divided into 5 groups. Of these, 56 were completely hypophysectomized at intervals varying from 8 days to 4 months prior to the death of the animal. 12 of these 56 had received daily subcutaneous injections of anterior lobe emulsion from the day of operation to the day of death. In 7 rats the posterior and part of the anterior lobe were removed. These animals were killed from 1 to 4 months after the operation. In 6 rats, the hypophysis was exposed but not removed. 20 control animals were included in this series.

physectomized 2 to 7 weeks before death was 0.213 per cent. In rats hypophysectomized 8 to 18 weeks before death the average ratio was 0.189 per cent. In rats that had received repeated injections of an emulsion of the anterior lobe during a period of 14 days prior to death and that had been hypophysectomized 14 to 30 days prior to the death of the animal, the average ratio of spleen weight to body weight was 0.33 per cent. The operative procedure itself, without removal of the hypophysis, had little effect on the size of the spleen. In those animals in which the hypophysis was partly removed from 1 to 5 months prior to death the results were very variable. The atrophy of the spleen was very marked in 3 instances in which the animals survived 5 months, even though a fragment of anterior lobe was present at autopsy. The other animals, killed within shorter periods, showed no atrophy in 2 instances and moderate atrophy in 2 instances. Whatever factor is responsible for spleen growth seems to be exhausted in spite of persistence of anterior hypophyseal fragments. (Text-figs. 1 and 2.)

Histologically, the spleen in hypophysectomized rats showed the picture of atrophy with a decrease in the size of the follicles though a relative increase in their number per unit area. There is an increase in the intersinusoidal connective tissue and a collapse of the sinuses. These are relatively bloodless. The Malpighian corpuscles are small. It is apparent from these observations that removal of the hypophysis in the rat results in a progressive atrophy of the spleen. After a period of 2 to 5 months the ratio of spleen weight to body weight is one-half the normal. This degree of atrophy may be as prominent as that of the atrophy of the thyroid or suprarenal glands. (Figs. 1 to 3.)

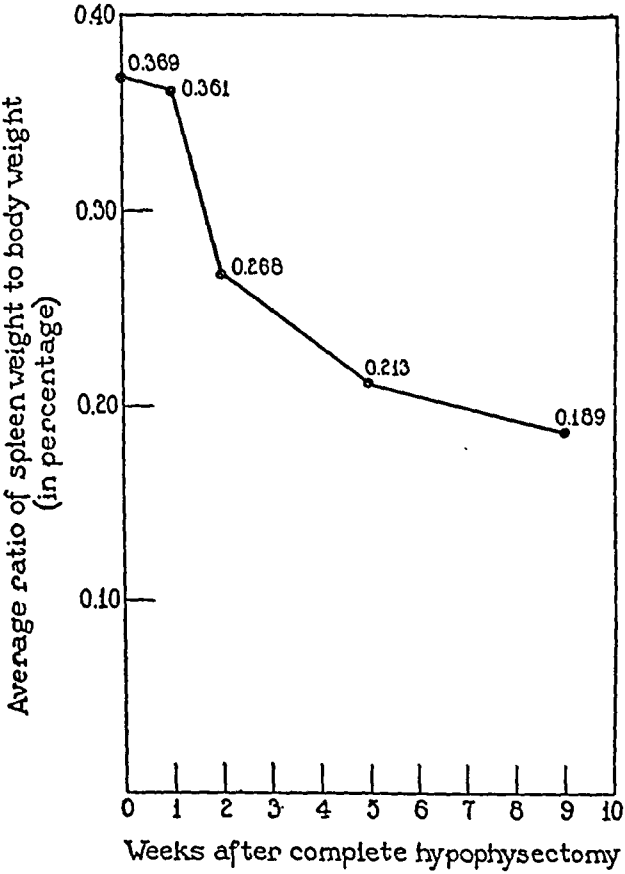
The Effect of Complete Hypophysectomy on the Regeneration of Splenic Tissue

An effort was made in a second group of experiments to determine the effect of complete and partial hypophysectomy on the regenerative capacity of splenic tissue in rats after removal of four-fifths of the spleen.[1] In addition, the course of splenic tissue regeneration in

[1] Anderson, Thomson and Collip (3) observed that removal of one suprarenal is not followed by hypertrophy of the other in the absence of the hypophysis. This failure of regeneration they attributed to the withdrawal of an "adrenotropic"

the spleen and the ratio of the spleen weight to body weight were determined. It was possible to estimate readily whether the loss of spleen weight was greater than or paralleled the loss in body weight.

As indicated in Table I, the average ratio of spleen weight to body weight of 20 normal rats of this stock, 3 to 4 months of age, was 0.37



TEXT-FIG. 2. The effect of complete hypophysectomy on the relative weight of the spleen. The curve indicates the progressive atrophy of the spleen at varying intervals after hypophysectomy. The points on the chart represent the average ratio of spleen weight to body weight in the given interval.

per cent. It will be observed that removal of the hypophysis was followed by a progressive decrease in the ratio of spleen to body weight. The average ratio of spleen weight to body weight in animals in which the hypophysis had been removed 1 week prior to death was 0.31 per cent. The average ratio of spleen weight to body weight in rats hypo-

physectomized 2 to 7 weeks before death was 0.213 per cent. In rats hypophysectomized 8 to 18 weeks before death the average ratio was 0.189 per cent. In rats that had received repeated injections of an emulsion of the anterior lobe during a period of 14 days prior to death and that had been hypophysectomized 14 to 30 days prior to the death of the animal, the average ratio of spleen weight to body weight was 0.33 per cent. The operative procedure itself, without removal of the hypophysis, had little effect on the size of the spleen. In those animals in which the hypophysis was partly removed from 1 to 5 months prior to death the results were very variable. The atrophy of the spleen was very marked in 3 instances in which the animals survived 5 months, even though a fragment of anterior lobe was present at autopsy. The other animals, killed within shorter periods, showed no atrophy in 2 instances and moderate atrophy in 2 instances. Whatever factor is responsible for spleen growth seems to be exhausted in spite of persistence of anterior hypophyseal fragments. (Text-figs. 1 and 2.)

Histologically, the spleen in hypophysectomized rats showed the picture of atrophy with a decrease in the size of the follicles though a relative increase in their number per unit area. There is an increase in the intersinusoidal connective tissue and a collapse of the sinuses. These are relatively bloodless. The Malpighian corpuscles are small. It is apparent from these observations that removal of the hypophysis in the rat results in a progressive atrophy of the spleen. After a period of 2 to 5 months the ratio of spleen weight to body weight is one-half the normal. This degree of atrophy may be as prominent as that of the atrophy of the thyroid or suprarenal glands. (Figs. 1 to 3.)

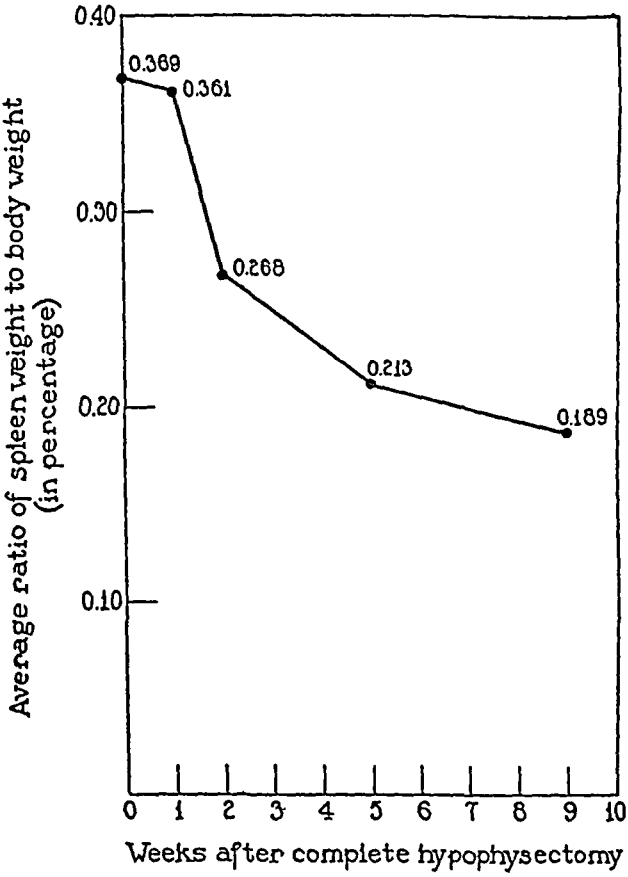
The Effect of Complete Hypophysectomy on the Regeneration of Splenic Tissue

An effort was made in a second group of experiments to determine the effect of complete and partial hypophysectomy on the regenerative capacity of splenic tissue in rats after removal of four-fifths of the spleen.[1] In addition, the course of splenic tissue regeneration in

[1] Anderson, Thomson and Collip (3) observed that removal of one suprarenal is not followed by hypertrophy of the other in the absence of the hypophysis. This failure of regeneration they attributed to the withdrawal of an "adrenotropic"

the spleen and the ratio of the spleen weight to body weight were determined. It was possible to estimate readily whether the loss of spleen weight was greater than or paralleled the loss in body weight.

As indicated in Table I, the average ratio of spleen weight to body weight of 20 normal rats of this stock, 3 to 4 months of age, was 0.37



TEXT-FIG. 2. The effect of complete hypophysectomy on the relative weight of the spleen. The curve indicates the progressive atrophy of the spleen at varying intervals after hypophysectomy. The points on the chart represent the average ratio of spleen weight to body weight in the given interval.

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The Presence of a Spleen-Stimulating Factor in Extracts of Anterior Hypophysis

(*a*) *The Effect of Repeated Injections of Extracts of Anterior Hypophysis of Cattle on the Size of the Spleen in Bartonella Carrier Rats*

In a third series of experiments the effect of injections of emulsions and extracts of cattle anterior hypophysis on the size of the spleen in normal adult rats was studied. This was undertaken to determine whether the anterior hypophysis contains a stimulating factor for splenic tissue and whether such a factor is present in the acid or alkaline extract of the anterior hypophysis.

62 rats, 3 to 4 months of age, were used in these experiments. 12 received daily subcutaneous injections of a saline emulsion of fresh cattle anterior hypophysis in amounts of 1 cc. during a period of 10 days. 7 rats received similar injections during a period of 30 days. 10 rats received similar injections during a period of 5 days. 24 hours after the last injection in each group the rats were killed with ether and the internal organs, including the spleen, were weighed. The ratio of spleen weight to body weight was determined.

6 rats received daily subcutaneous injections of an acid extract of acetone-dried anterior hypophysis (Loeb's method) in amounts of 1 cc.[3] during a period of 10 days. The spleen and other organs were weighed at the end of that period as in the previous groups.

7 rats received daily subcutaneous injections of a purified growth fraction (prepared according to the method of Collip (6) from fresh anterior hypophysis in amounts of 1 cc. during a period of 10 days. This fraction was found to contain considerable gonadotropic hormone as well.

As controls for spleen weight in our stock we used 20 normal rats of the same age group as the above.

The influence on the spleen weight of these injections is indicated in Table III. The average ratio of spleen weight to body weight, expressed in percentage, of rats of *Bartonella* carrier stock, 3 to 4 months of age, is 0.37. Rats injected with saline emulsion of fresh cattle anterior hypophysis during 5 days show a definite increase in the absolute and relative size of the spleen (0.56 per cent of the body weight). When the emulsion is administered during a period of 10 days, the spleen becomes two and one half times the normal size (the ratio of spleen weight to body weight, 0.80). It is of considerable

[3] An attempt was made to give extracts in equivalent estimated amounts of fresh gland material in the various groups.

TABLE II—*Concluded*

Rat No.	Approximate weight† of spleen stump at time of partial removal of spleen	Weight of spleen stump at death	Difference in weight of spleen stump at operation and death	Increase in weight of spleen stump	Ratio of weight of spleen stump to body weight at operation	Ratio of weight of spleen stump to body weight at death	Difference between ratio of weight of spleen stump to body weight at death and operation
			Controls				
17	94	171	+77	81.9	0.063	0.098	+0.035
18	54	91	+37	68.5	0.034	0.067	+0.033
19	44	132	+88	200	0.025	0.077	+0.052
20	47	113	+66	140.4	0.024	0.071	+0.047
21	119	197	+78	65.5	0.068	0.082	+0.014
Average.....				+111.26			+0.036

per cent. In rats in which the posterior lobe and part of the anterior lobe of the hypophysis had been removed, the spleen tissue regenerated but the degree of regeneration varied considerably. The difference between the ratio of weight of the spleen stump to the body weight at operation and at death in the 4 groups of animals showed corresponding results (see Table II).

The histological picture of the regenerated splenic stump in the normal animal 30 days after partial splenectomy shows an increase in the reticular cells of the red pulp with numerous islands of small lymphocytes. Otherwise the splenic stump has all the appearances of a normal spleen. The histological appearance of the splenic stump in animals that had been hypophysectomized previous to partial splenectomy shows a marked atrophy of the follicles and a definite decrease in the reticular tissue of red pulp.

The data presented in these experiments suggest that the anterior lobe of the hypophysis is essential for splenic tissue regeneration. In the absence of the hypophysis, removal of four-fifths of the spleen is not followed by increase in size of the remaining stump. The administration of emulsion of fresh anterior hypophysis in hypophysectomized rats restores the regenerative capacity of splenic tissue to the normal level.

to control the effect of injections of protein as such upon the spleen. Rich, in a recent report (8), mentions that the spleen of rabbits may enlarge, following the repeated injection of nonbacterial antigenic material, egg white or horse serum for example.

In order to determine what part a nonspecific effect of protein played in the increased size of the spleen in our experiments, the effect of horse serum and of extracts of organs other than the hypophysis was studied.

All the rats in these experiments were of Wistar stock, free from *Bartonella* infection, and all were 8 to 9 weeks of age at the time the injections were commenced. In each instance the extracts were administered daily during a period of 10 days and the rate of growth and size of spleen determined. The following extracts were used: (*a*) saline emulsion of fresh anterior hypophysis of cattle, (*b*) saline emulsion of fresh anterior hypophysis of pig, (*c*) alkaline extract of acetone-dried anterior hypophysis of cattle, (*d*) an alkaline extract of acetone-dried kidney tissue of cattle, (*e*) an alkaline extract of acetone-dried liver tissue of cattle, (*f*) an alkaline extract of acetone-dried spleen tissue of cattle, and (*g*) an acid extract of acetone-dried anterior hypophysis of cattle.

In each test, 6 rats were used. There was a striking consistency in spleen weights in each group. The spleen weights of normal controls of this stock and those given in Donaldson's figures were used for comparison. The results are indicated in Table IV. The average ratio of spleen weight to body weight in normal *Bartonella*-free rats of Wistar stock of this age as determined by the control group and as calculated from Donaldson's tables is approximately 0.27 per cent. It will be observed from the table that the repeated injections of horse serum and alkaline extracts of kidney, liver and spleen prepared in the manner of the hypophyseal extracts had little or no effect on the size of the spleen. The saline emulsions of anterior hypophysis and, notably, the alkaline extract of the dried anterior hypophysis had the most pronounced effect on the growth of the spleen, as in the earlier experiments with *Bartonella* carrier rats. The average ratio of spleen weight to body weight in rats injected with such alkaline extracts was 0.486 (almost twice that of the controls).

The preliminary attempt at separation of the spleen-stimulating factor suggests its presence in the alkaline fraction of the dried, as well as of the fresh hypophysis, and its absence in the acid fraction of the dried gland. It is important that in the animals tested (which were

TABLE IV

*The Effect of Repeated Injections of Anterior Hypophyseal Extracts of Cattle on the Size of the Spleen in Bartonella-Free Rats**

All Extracts Were Injected Daily during a Period of 10 Days in Amounts of 1 Cc.

No. of rats	Material injected	Ratio of spleen weight to body weight Average	Ratio of spleen weight to body weight Average Calculated from Donaldson's tables†	Increase in body weight Average
		per cent	*per cent*	*per cent*
6	Saline emulsion of fresh anterior hypophysis (cattle)‡	0.37	0.270	38
6	Saline emulsion of fresh anterior hypophysis (pig)	0.392	0.264	54
6	Alkaline extract (crude) acetone-dried anterior hypophysis§	0.486	0.267	27.4
6	Alkaline extract acetone-dried kidney (cattle)‖	0.250	0.269	25.3
6	Alkaline extract acetone-dried liver (cattle)‖	0.278	0.267	24
6	Alkaline extract acetone-dried spleen (cattle)‖	0.290	0.273	30
6	Acid extract acetone-dried anterior hypophysis (cattle)**	0.294	0.271	45
6	Horse serum	0.272	0.269	28
10	None	0.270	0.272	26.5
	Donaldson's figures for Wistar rats. Calculated expected ratio of weight of spleen to body weight. Average.....		0.269	

* All rats were of Wistar stock, 8 or 9 weeks of age. In each group equal numbers of male and female rats were used.

† The expected weight of the spleen of rats of Wistar stock may be calculated from the body weight by the following formula (9): 0.00245 body weight + 0.0301 log (body weight) − 0.025.

‡ The saline emulsion of fresh anterior hypophysis was made by emulsifying the anterior lobes of 8 glands in 80 cc. of saline. It was freshly prepared with aseptic technique twice a week.

§ The alkaline extract was prepared as follows: 10 gm. of dried powder was extracted with 100 cc. of 0.5 N sodium hydroxide overnight. The fluid was brought up to pH 7.8, centrifuged and filtered through the Zeiss filter.

‖ The organ extracts were prepared exactly like the alkaline hypophyseal extract (see §). The acetone-dried tissue was obtained through the courtesy of Dr. David Klein of the Wilson Laboratories.

** The acid extract of dried anterior hypophysis was prepared by method of Loeb (5). 10 gm. of dried powder was extracted with 100 cc. of ½ per cent acetic acid overnight. The fluid was brought to pH 6.8, centrifuged and then brought to 7.8 and filtered through a Zeiss filter.

affected.[4] Sections through the lymph nodes show some hyperplasia of the follicles. The Kupffer cells of the liver are unaffected. (Figs. 4 to 7).

DISCUSSION

The evidence presented in this communication supports the view that there is a relationship between the anterior lobe of the hypophysis and the spleen. The complete removal of the hypophysis in the adult rat is followed by a progressive atrophy of the spleen which exceeds the rate of loss in weight of the animal. The degree of atrophy is analogous in many instances to that of suprarenals or thyroid. The atrophy of the spleen is dependent on the withdrawal of a stimulating factor in the anterior hypophysis, since removal of the posterior lobe and part of the anterior is not followed by a similar degree of atrophy of the spleen. The injection of a saline emulsion of fresh anterior hypophysis in hypophysectomized rats during a period of 14 days prior to death, repairs in most instances the atrophy of the spleen.

The inhibitory effect of hypophysectomy on the regenerative capacity of splenic tissue is further evidence of the presence of a controlling factor in the anterior hypophysis. In the normal adult rat, removal of four-fifths of the spleen is followed by hypertrophy of the stump to about one-third the normal spleen. In the hypophysectomized rat the stump becomes absolutely and relatively smaller within the same period of observation. The capacity for regeneration of the splenic tissue in hypophysectomized rats is restored by the administration of anterior hypophyseal emulsion.

The anterior hypophysis possesses a factor capable of stimulating the growth of the spleen in the normal rat. This factor is not present in the acid extract of anterior hypophysis but is present in a marked degree in the alkaline fraction containing the growth and gonadotropic hormones. An alkaline extract of dried anterior hypophysis containing little or no growth factor is rich in the spleen-stimulating factor. As in the case of hypophyseal hormones, a tolerance develops in the normal rat to its injection and the maximal splenic effect is attained within 2 weeks. Little effect is apparent after a month of daily administration (antihormone effect?).

[4] No significant increase in the circulating blood cells was observed.

still immature) the alkaline extract of the dried gland yielded the maximal spleen-stimulating effect, but the rate of growth in these animals was normal. The alkaline extract of dried powder is a relatively poor source of growth hormone but an excellent source of the spleen-stimulating factor. The acid extract of the powder contained no spleen-stimulating factor but was a fairly good source of growth hormone.

It would seem then that the spleen-stimulating factor exists in the alkaline extract of the fresh or dried anterior hypophysis but is probably distinct from growth hormone. These experiments further suggest that the anterior hypophysis contains a spleen-stimulating factor and that the effect of its injection is not due to the repeated injection of antigenic protein material, nor are similar effects observed when other organ extracts are injected.

Histological Changes Induced by Anterior Hypophyseal Extracts

The spleens of rats injected with fresh anterior hypophysis emulsion or an alkaline extract of fresh or dried anterior hypophysis show a striking histological appearance. There is a hyperplasia of the germinal centers of the follicles. The outer zone of mononuclear cells (modified reticular cells) is much wider than normal and fades into the red pulp. There is a pronounced proliferation of reticular and endothelial cells throughout the red pulp. Aggregates of these cells appear which contain small clusters of small lymphocytes. There is an increase in the number of megakaryocytes. Considerable hemosiderin pigment is present. Histological appearance of spleen in those animals that have been injected with the acid extract of the anterior hypophysis (thyrotropic fraction) is the same as that observed in the normal controls. The repeated injection of horse serum seems to have some effect on the cellular elements in the spleen. Some increase in the outer zone of mononuclear elements of the follicles occurs. Changes, however, do not occur in the red pulp, such as are observed when the hypophyseal emulsion or alkaline extract is used.

Repeated injections of fresh emulsion of the anterior hypophysis or of alkaline extracts were associated with a great stimulation in the number of hemocytoblasts of the bone marrow. The number of megakaryocytes is also largely increased. Erythropoiesis seems un-

is also present in alkaline extracts of acetone-dried anterior hypophysis relatively free from growth hormone.

6. The presence of a spleen-stimulating factor in the anterior hypophysis is suggested by these experiments.

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EXPLANATION OF PLATES

PLATE 43

FIG. 1. Photographs of the spleens of (*a*) normal rats treated with anterior hypophyseal emulsion during a period of 10 days, (*b*) normal rats and (*c*) completely hypophysectomized rats (1 month after operation). About natural size.

FIG. 2. Photographs of splenic stumps (*a*) in normal rats, (*b*) in hypophysectomized rats treated with anterior hypophyseal emulsion and (*c*) in untreated hypophysectomized rats. Four-fifths of the spleen was removed 4 weeks prior to death in all the rats. Hypophysectomy was done 1 month prior to partial splenectomy. About natural size.

FIG. 3. Spleen 73 days after hypophysectomy showing decrease in size of follicles and relative increase in number per unit area. ×70.

PLATE 44

FIG. 4. Spleen of normal rat of same age. ×70.

FIG. 5. Spleen of rat repeatedly injected with horse serum. The follicles are increased in size. ×70.

PLATE 45

FIG. 6. Spleen of rat repeatedly injected with an alkaline extract of cattle anterior hypophysis. Note the marked hyperplasia of follicles and increase in reticular cells about the margin. ×70.

FIG. 7. Spleen of rat repeatedly injected with emulsion of anterior hypophysis. Hyperplasia of follicles, particularly large lymphoid elements and reticular cells. Striking increase in reticular cells of the pulp with ectopic foci of lymphocytes. ×70.

It would seem probable that a spleen-stimulating factor or hormone in the anterior hypophysis may exist. Though the evidence is suggestive at present, it cannot be stated with certainty that the spleen-stimulating factor is separable from the growth and gonadotropic principle.

SUMMARY

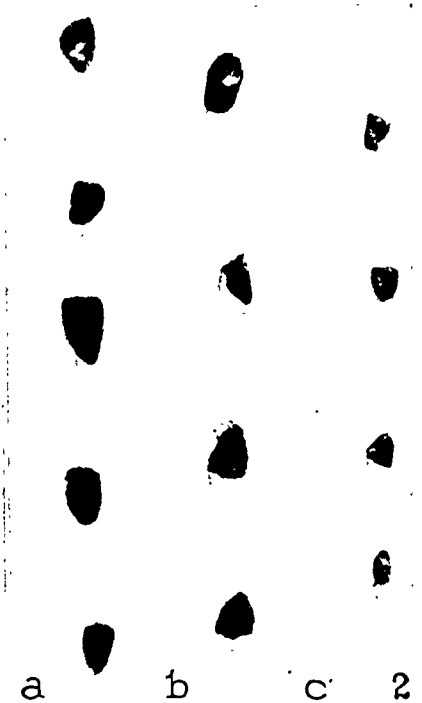
1. Removal of the hypophysis in adult rats is followed by progressive atrophy of the spleen. At the end of 2 months the ratio of spleen weight to body weight is one-half the normal. The administration of hypophyseal emulsion repairs to a considerable degree the atrophy of the spleen in such animals.

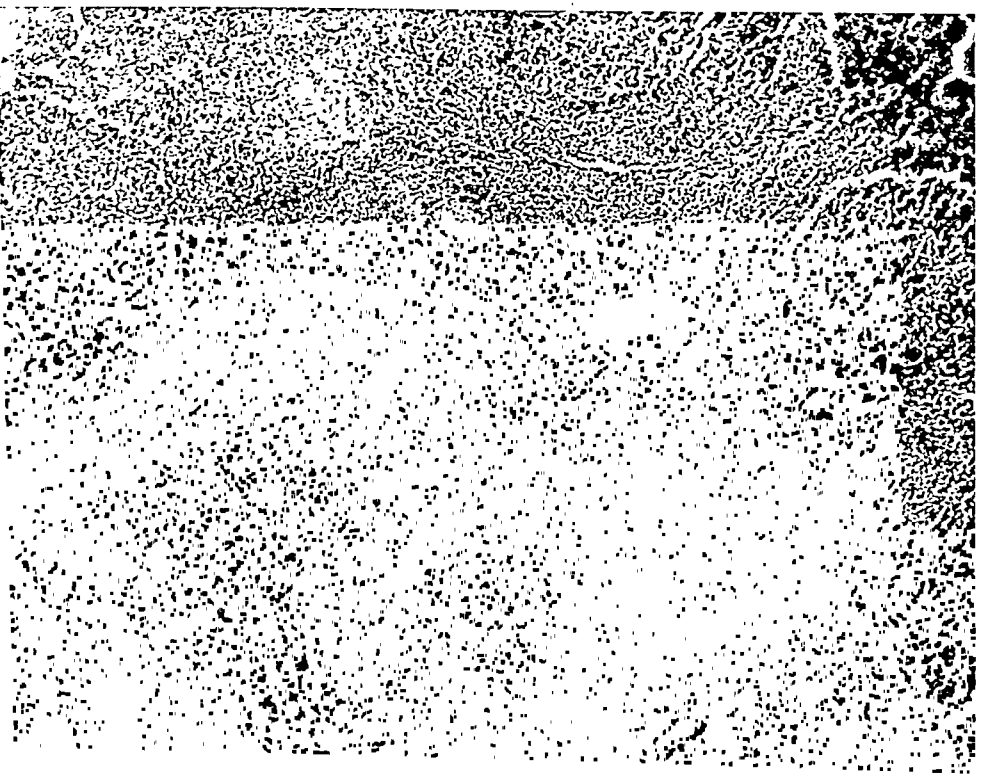
2. Hypophysectomy completely inhibits the regeneration of splenic tissue after partial splenectomy. Administration of anterior hypophyseal emulsion restores the regenerative capacity of splenic tissue of the hypophysectomized rat to the normal.

3. The daily administration of anterior hypophyseal emulsion of cattle or of alkaline extracts of fresh or acetone-dried anterior hypophysis during a period of 10 days in normal *Bartonella* carrier or *Bartonella*-free rats results in hypertrophy of the spleen to twice normal size. Normal rats receiving emulsion during a period of 1 month become refractory to the spleen-stimulating effect. The spleen shows little increase in size above the normal at the end of this period. Injections of horse serum or of alkaline extracts of acetone-dried kidney, spleen or liver of cattle did not cause enlargement of the spleen of rats of *Bartonella*-free stock.

4. The increase in the size of the spleen, following daily administration of emulsion of the anterior hypophysis, is due primarily to a marked hyperplasia of the reticular and endothelial cells of the red pulp. The follicles also increase in size. Clusters of reticular cells containing numerous lymphoid elements appear throughout the splenic pulp. The reticular tissue of the bone marrow is similarly increased. There is a striking increase in the number of hemocytoblasts and megakaryocytes. The Kupffer cells are not affected.

5. The spleen-stimulating factor is not present in the acid extract of anterior hypophysis that contains thyrotropic and adrenotropic factors. It is present in some degree in an alkaline extract of fresh anterior hypophysis containing growth and gonadotropic factors. It





(Perla: Relation of hypophysis to spleen I and II)

THE EFFECT OF COMBINATION WITH DIAZO COMPOUNDS ON THE IMMUNOLOGICAL REACTIVITY OF ANTIBODIES

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Previous investigations into the chemistry of antigen-antibody reactions have dealt largely with antigens. The effect of chemical modification on the immunological reactivity of protein antigens has been extensively studied by Landsteiner and his coworkers; and considerable progress has been made in the chemical identification of the antigenic constituents of bacteria, red blood cells and other complex cellular antigens. In comparison, little is known of the chemistry of antibodies. There is a considerable body of evidence which indicates with a strong degree of probability that antibodies are proteins associated with the globulin fraction of the serum which combine with the homologous antigen to form a surface layer of protein, somehow rendered insoluble by this combination. These conclusions are supported by the chemical properties of the antibody in solution, the surface properties of antigen-antibody compounds, their flocculating tendencies and immunological behavior, and by the N content of antigen-antibody aggregates.[1] Little has been done, however, to identify either the chemical groups in antibodies which endow them with their specific reactivity, or those which are responsible for the characteristic properties of antigen-antibody compounds: susceptibility to aggregation and to phagocytosis, and the extraordinary avidity for complement which results in complement fixation, hemolysis, bacteriolysis and bactericidal action. The

[1] Literature summarized by Marrack; Heidelberger; Mudd, Lucké, McCutcheon and Strumia; Eagle (1930).

several animal species, giving agglutination, precipitation and complement fixation, animal protection, hemolysis and hemagglutination with the homologous antigens; and these antigens included bacteria, red cells, carbohydrate, toxin, protein and lipoid.

Coupling of the Antisera with the Diazo Compound.—The experiments were greatly facilitated by a technic which allowed the reaction between the diazo compound and the serum to be terminated after any desired interval. By taking aliquot samples, it was possible to follow the progressive change in the reactivity of the antibody as more and more groups combined with the diazo compound. Essentially the same method was used throughout.

The antiserum and *e.g.* diazotized and neutralized sulfanilic acid were mixed in the proportions indicated in the various tables. Samples were withdrawn at intervals ranging from 15 seconds to 24 hours, and to each sample was added a M/7 solution of Na sulfanilate or metanilate in twice the quantity necessary to combine with the diazo compound originally present in the sample. As shown by tests with α-naphthol, any excess diazo compound combined rapidly and completely (>99.9 per cent)[4] with the sulfanilate; and such a mixture usually remained unchanged as regards antibody activity after 24 hours at room temperature or at 37°C. Occasionally, however, a mixture of Na sulfanilate and diazosulfanilate caused a slight but definite decrease in the antibody activity of dilute antiserum.

Diazotized Sulfanilic Acid.—For 200 cc. of a M/7 solution (the amount usually prepared at one time), 5 gm. of sulfanilic acid were treated with 1.75 gm. of anhydrous Na_2CO_3 dissolved in 75 cc. of water, and with 2.1 gm. of $NaNO_2$ dissolved in 10 cc. of water. The solution was cooled in ice water and diazotized at 3–5°C., using a mechanical stirrer, by the slow addition of 35 cc. of N/1 HCl from a separatory funnel, the tip of which was immersed in the liquid. The pale yellow, somewhat acid diazonium solution was brought to pH 7.2–7.6 with N/1 NaOH. Since the solutions coupled with the pH indicators ordinarily used, it was found necessary to prepare a somewhat yellowish solution of bromthymol blue and note the evanescent color formed on adding a few drops of this to a little of the diazo solution contained in a small tube. 8 cc. of 0.5 M phosphate buffer containing 4 parts of Na_2HPO_4 to one part of KH_2PO_4 were then added, and the whole diluted to 200 cc.

Other Diazo Compounds.—Diazotized arsanilic, *p*-aminobenzoic, 1-amino-2-

[4] The diazo solution originally gave a brilliant red color in a 1:8000 dilution on testing with α-naphthol. Within 1 minute after the addition of the sulfanilate, a 1:10 dilution gave only a trace of color, and within 5 minutes, the reactivity with α-naphthol had almost completely disappeared.

present paper, dealing with the effect of various diazo compounds on the reactivity of antibodies, represents an attempt in that direction. The groups in protein which react with diazo compounds are not only the imidazole ring of histidine and the phenyl group of tyrosine (a phenolic derivative) as described by Pauly, but probably include the aliphatic NH_2 groups, the proline and hydroxyproline NH group, and the indole group of tryptophane (Eagle and Vickers). The immunological reactivity of the "coupled" antibody globulin should therefore provide a clue to the extent to which these groups in antibody participate in its combination with antigen, and the extent also to which they are responsible for the characteristic properties of the antigen-antibody complex.

Breinl and Haurowitz have found that antibodies are completely destroyed by diazotized aniline, atoxyl or metanilic acid. Reiner, however, as well as Bronfenbrenner, Hetler and I. O. Eagle, report little or no destruction of horse serum antibodies despite the complete loss of their species specificity.[2] As will appear in the following, the apparent discrepancy is due to differences in the degree of coupling; and particular interest attaches to the partially inactivated antibodies of peculiarly modified reactivity to be here described.

Methods and Materials

The antisera studied were horse antipneumococcus serum and refined globulin; horse diphtheria antitoxin serum and globulin; horse and rabbit antityphoid sera; rabbit antisera *vs.* sheep red blood cells, horse serum, and egg albumen; and the reagin of Wassermann positive human sera.[3] There were thus included representative sera from

[2] That is, the coupled horse antibody preparations no longer reacted immunologically as horse serum protein.

[3] We are indebted to the Mulford Laboratories, Glenolden, Pennsylvania, for their generous cooperation in furnishing large quantities of antipneumococcus serum, diphtheria toxin and antitoxin, and horse antityphoid serum for use in these studies; to the Massachusetts State Antitoxin Laboratory for their kindness in supplying antipneumococcus serum; to Dr. Walther Goebel, of The Rockefeller Institute for Medical Research, for a generous supply of Type I and II pneumococcus carbohydrate, and to the Wassermann laboratories of The Johns Hopkins Hospital, Baltimore, and the Graduate Hospital, Philadelphia, for normal and Wassermann positive human serum.

inhibited the flocculating tendency of the antitoxin, without affecting its combining affinity for toxin. Similar results were obtained with

TABLE I

The Effect of Diazotized Sulfanilic Acid on the Immunological Reactivity of Horse Diphtheria Antitoxin

2 cc. of the N/7 diazo solution were added to 6 cc. of antiserum. 1 cc. samples were withdrawn at the stated intervals and 0.5 cc. of N/7 Na metanilate were added. The controls consisted of 0.5 cc. serum mixed with 0.5 cc. of either N/7 NaCl or a 2:1 metanilate:diazo mixture.

Duration of coupling	Degree of flocculation* 2 cc. toxin and varying quantities of the coupled serum						Guinea pig protection experiments with 0.2 cc. toxin and varying quantities of the coupled serum					Conclusion
	0.2 cc.	0.15 cc.	0.1 cc.	0.075 cc.	0.05 cc.	0.038 cc.	0.2 cc.	0.05 cc.	0.125 cc.	0.0062 cc.	0.0031 cc.	
min.												
0	Cl*	Cl	4	4	4	0	S†	S	S	S	D2	Flocculating activity completely destroyed in 4 to 16 minutes; approximately 50% of protection activity remains after 24 hrs.
1	0	0	Cl	2	2	0	S	S	S	S	D2	
4	0	0	0	Cl	0	0	S	S	S	S	D2	
16	0	0	0	0	0	0	S	S	S	S	D1	
64	0	0	0	0	0	0						
250	0	0	0	0	0	0	S	S	S	D2	D1	
Overnight	0	0	0	0	0	0	S	S	S	D2	D1	
Overnight coupling with twice as much diazo compound	0	0	0	0	0	0	D1	D1	D1	D1	D1	Complete destruction of antibody on sufficient coupling
Control with inactivated diazo solution	Cl	Cl	4	4	4	0	S	S	S	S	D2	Antibody unaffected by inactivated diazo compound

* Cl = cloudy after 24 hours at 37°C.
2,4 = degrees of precipitation after 24 hours at 37°C.
0 = no visible aggregation.
† S = survived > 4 days.
D1 = dead in 1 day, etc.

naphthol-4-sulfonic and 4-aminotoluene-2-sulfonic acids were prepared in essentially the same manner.

Titration of the Antibody Content of the Several Sera.—(*a*) The original serum, (*b*) a control containing serum incubated with an inactive mixture of the Na sulfanilate and diazosulfanilate and (*c*) samples of serum allowed to react with the diazo compound for varying periods of time before the addition of Na sulfanilate to terminate the reaction, were all tested for antibody content by the methods to be described in the following pages.

Diphtheria Antitoxin

The biological activity of diphtheria antitoxin can be tested either by observing the flocculation obtained on the addition of toxin (Ramon), in which case the serum:toxin ratio giving the most rapid flocculation is the index of the potency of the antitoxin, or by determining the minimum amount of serum which protects guinea pigs against some arbitrarily chosen multiple of the lethal dose of toxin. Preliminary tests with twenty-three antitoxic sera and ten lots of toxin from seven different laboratories indicated that, as reported by Ramon, Bayne-Jones, and others, and contrary to statements in the literature, the two methods of titration yield essentially similar results. The ratio of the protective titer:flocculation titer in these twenty-three sera was 1.02 ± 0.04, indicating an almost exact correlation.

Despite this parallelism, and despite the probable identity of the serum constituent giving these two reactions, the addition of Na diazosulfanilate caused a complete dissociation between the flocculating and protective properties of diphtheria antitoxin. One of six experiments yielding qualitatively similar results is summarized in Table I. The flocculating activity of the serum was definitely impaired within a short time after the addition of the diazo compound, in some experiments after as little as 30 seconds. This impairment consisted in a marked prolongation of the time required for flocculation. After a few minutes coupling, it no longer gave a definite precipitate, even after overnight incubation with toxin, but became diffusely cloudy; and on longer coupling, the antitoxin no longer reacted in any visible manner with toxin. However, the protective titer of the serum as measured in guinea pigs remained at its original level long after its flocculating activity had thus been completely inhibited. The introduction of a few diazo molecules had completely

Antipneumococcus Horse Serum

Four immunological reactions given by antipneumococcal sera were available for study: bacterial agglutination, the precipitation given with the type-specific carbohydrate derived from the bacterial capsule, complement fixation with a bacterial suspension and the protection of mice against multiple lethal doses of pneumococcus culture.

The agglutinating titer was determined by the following technic, using heat-killed 24 hour cultures of Type I organisms grown on 0.2 per cent glucose infusion broth, washed and concentrated to 1/5 the original culture volume.

Serum, *cc.*	0.8	0.4	0.2	0.1	etc.
0.85 per cent NaCl, *cc.*		0.4	0.6	0.7	etc.
Bacterial suspension, *cc.*	0.2	0.2	0.2	0.2	etc.

The precipitating titer of the serum against the deacetylated Type I and Type II capsular carbohydrates was similarly determined, using a 0.01 per cent solution of the carbohydrate instead of the bacterial suspension. In the protection experiments, 0.1 cc. of a 24 hour culture of the organisms in blood broth was injected intraperitoneally, and varying quantities of the serum sample to be tested were injected immediately into the opposite side of the peritoneal cavity. The animals were then observed over a 4 day period.

The diazotized sulfanilic acid caused a complete dissociation of these several properties, this despite the fact that they are reported to be due to the same antibody (Felton). The three experiments summarized in Table II are illustrative of ten others with three different sera and two different globulin preparations, and are diagrammatically summarized in Fig. 1. Qualitatively the same results were obtained with all of the other diazo compounds tested (page 620).

The first effect produced on the antipneumococcus serum (or concentrated globulin) by the Na diazosulfanilate was a disappearance of its ability to give precipitation with the specific soluble substance. This inactivation began to be apparent within a few minutes after the diazo compound was added to an equal volume of serum, and long before there was any change in either the agglutinating, complement fixing or protective titer of the serum.

As coupling proceeded, these also began to be affected. The bacterial aggregates became smaller, the characteristic large coherent

diazotized preparations of arsanilic acid, *p*-aminobenzoic acid, 1-amino-2-naphthol-4-sulfonic acid and 4-aminotoluene-2-sulfonic acid.

It is noteworthy that the progressive retardation of the flocculating activity of the serum as coupling proceeded was not associated with any significant change in the serum:toxin ratio giving the most rapid flocculation or turbidity. This optimum ratio, which is taken as the index of the serum antitoxin content, remained constant, and only the time required for flocculation or clouding to become apparent was affected. This constitutes strong evidence that all of the antitoxin was being simultaneously affected by the diazo compound (page 637).

It becomes of interest to ascertain whether the partially coupled antibody fails to flocculate on the addition of toxin because it can no longer combine with toxin, or whether, as suggested by the *in vivo* protection experiments, combination proceeds normally, and only the secondary aggregation of the toxin-antitoxin compound is inhibited. The following experiment indicates that the latter is the case. If one adds 1 L + dose of toxin to a neutralizing quantity of partially coupled antibody, and then precipitates the latter with a rabbit antiserum to horse serum, the supernatant fluid is found to be non-toxic, showing that the toxin had been precipitated along with the antitoxin. A similar experiment carried out with normal horse serum instead of partially coupled antitoxin, or with antitoxin so completely coupled that it had lost its ability to neutralize toxin *in vivo*, results in a supernatant fluid of undiminished toxin content (Eagle, 1935 *a*). It would therefore appear that partially coupled antitoxin neutralizes toxin by virtue of the fact that it can still combine with toxin; but the secondary aggregation (Ramon flocculation) is somehow inhibited by the reaction of the diazo compound with the antibody molecule.

The puzzling observation that pseudoglobulin concentrated from antitoxin sera may fail to give the Ramon flocculation reaction, despite the fact that it combines with toxin *in vitro* (Eagle, 1935 *a*) and retains its protective action *in vivo*, may well be due to some analogous chemical modification produced in the course of its salting out and subsequent dialysis. It would appear from the preceding experiments with diazo compound that only a few groups in the antibody molecule need be modified in order to inhibit flocculation completely.

						1.6 cc.	0.4 cc.	0.1 cc.	0.05 cc.	0.25 cc.
Experiment 3 6 cc. serum + 6 cc. of diazo solution. Aliquot samples of 1.5 cc. were withdrawn at intervals and the reaction terminated by the addition of 1.5 cc. Na metanilate	Overnight control with N/7 NaCl	4 4 4 4 2	4 4 4 4 2	4 4 1 ± 0 0			S	—	S	D2
	Overnight control with inactive diazosulfanilate: metanilate mixture	4 4 4 4 2	4 4 4 4 1	4 4 1 ± 0 0						
	1 min. coupling	4 4 4 4 1	4 4 4 4 1	2 2 ± 0			S	S	D2	D1
	4 " "	4 4 4 4 ±		1 ± 0 0			S	S	D3	D1
	16 " "	4 4 4 4 0		0 0 0 0 0 0		S	S	D2	D1	
	64 " "	4 4 4 3 0		0 0 0 0 0 0		S		S	D1	
	240 " "	4 4 4 2 0	4 4 4 4 0	0 0 0 0 0 0		S	D2	D2		
	Overnight "	4 4 2 0 0	4 4 4 2 0	0 0 0 0 0 0		D1	D1	D1		
	Overnight coupling with twice as much diazo solution	0 0 0 0 0	0 0 0 0 0	0 0 0 0 0 0						

* *Cf.* text, page 626.

† ac = anticomplementary.

TABLE II

The Effect of Coupling with Diazotized Sulfanilic Acid on the Immunological Reactivity of Antipneumococcus Serum and Globulin

	Duration of treatment	Reactivity of the treated antisera				
		1 Agglutination (4 hrs. at 37°)	2 Centrifuge agglutination*	3 Precipitation with SSSI	4 Complement fixation with bacteria	5 Mouse protection
Experiment 1						
4 cc. of 1:2 serum + 4 cc. of diazo solution. Aliquot samples of 1 cc. were withdrawn at intervals and the reaction terminated by the addition of 1 cc. Na metanilate	Overnight control with N/7 NaCl	4 4 4 1 ±	4 4 4 4 1	4 4 4 4 1 2	4 4 4 4 4 2	
	Overnight control with inactive diazosulfanilate: metanilate mixture	4 4 4 1 ±	4 4 4 4 1	4 4 4 4 1 0	ac† ac 4 4 4 2	
	1 min. coupling	4 4 4 1 ±	4 4 4 4 1	0 1 1 1 0 0	ac ac 4 4 4 2	
	4 " "	4 4 3 1 0	4 4 4 2 0	0 0 1 ± 0 0	ac ac 4 4 2 0	
	8 " "	4 4 2 1 0	4 4 3 3 0	0 0 1 ± 0 0	ac ac 4 4 2 0	
	60 " "	4 1 1 0 0	4 3 2 1 0	0 0 1 0 0 0	ac ac 4 2 0 0	
	Overnight "	0 0 0 0 0	0 0 0 0 0	0 0 0 0 0 0	ac ac ac ac 0 0	
Experiment 2						
15 cc. of 1:2 serum + 5 cc. of diazo solution. Aliquot samples of 3 cc. were withdrawn in intervals and the reaction terminated by the addition of 1.5 cc. Na metanilate	Overnight control with N/7 NaCl	4 4 4 4 ±	4 4 4 4 ±	4 4 4 4 0 0		
	Overnight control with inactive diazosulfanilate: metanilate mixture	4 4 4 4 4	4 4 4 4 4	4 4 4 4 2 0		
	½ hr. coupling	4 3 3 3 1	4 4 4 4 0	4 4 2 0 0 0		
	1½ hrs. "	3 2 1 0 0	4 4 4 4 0	± ± 0 0 0 0		
	2 " "	2 0 0 0 0	2 4 4 4 0	0 0 0 0 0 0		
	4 " "	± 0 0 0 0	4 4 4 ± 0	0 0 0 0 0 0		
	8½ " "	0 0 0 0 0	4 4 4 0 0	0 0 0 0 0 0		
	23 " "	0 0 0 0 0	4 4 3 0 0	0 0 0 0 0 0		

protein on the surface of the bacteria was not as cohesive as a similar deposit of unaltered antibody. The impacts between bacteria caused by their Brownian movement no longer resulted in cohesion, and it required the intimate pressure packing caused by the centrifuge to produce visible agglutination.

Coupling with the diazo compound beyond this stage caused a complete destruction of antibody; the protective action, complement fixing activity and the centrifuge agglutination test eventually disappeared completely. Usually, but not invariably, these reactions disappeared in the order named.

There are several possible explanations for these progressive changes in the reactivity of antipneumococcal sera.

1. The failure to precipitate might conceivably be due to the fact that partially coupled antibody loses its combining affinity for carbohydrate, or, equally likely, the coupled antibody may combine with carbohydrate, but fail to precipitate, just as partially coupled diphtheria antitoxin combines with toxin but fails to flocculate. However, the experiments described in Protocol 1 and illustrated in Table III indicate that the partially coupled antibody actually fails to combine with the carbohydrate, despite the fact that it can still cause bacterial agglutination.

Protocol 1

Experiments indicating that the failure of the partially coupled antibody to give precipitation with Type I and Type II pneumococcus polysaccharide is due to the absence of combination.

Varying quantities of diazotized sulfanilic acid were added to antipneumococcus serum, and the mixtures allowed to interact overnight in the ice box. The excess diazo compound was then neutralized by the addition of sulfanilic acid and the reactivity of the several solutions tested by adding Type I and Type II carbohydrate to varying quantities of the serum as indicated in Table III. Precipitation was read after 4 hours at 37°C. followed by overnight in the ice box. The supernatant fluid was then tested for excess antibody and excess carbohydrate by the addition of fresh carbohydrate and serum respectively. The quantities of serum and carbohydrate in the original mixtures were so chosen that if combination occurred, it would become apparent in the disappearance of free carbohydrate in some of the tubes of each series. A single experiment with Type I carbohydrate is given in Table IV and is illustrative of four similar experiments with both Type I and Type II carbohydrates. The results can be briefly sum-

flakes no longer formed and the agglutinating titer as read after 4 hours at 37°C. began to decrease. Generally, however, the precipitating activity with carbohydrate had been completely destroyed at a time when the serum still gave active agglutination, complement fixation and protection to almost the same degree as the original serum. At this intermediate stage, the partially coupled serum therefore resembled that often produced in rabbits by the injection of pneumococci, in so far as it gave all the reactions of a horse antiserum except the precipitation with specific soluble substance. The dissociation between carbohydrate precipitation and bacterial agglutina-

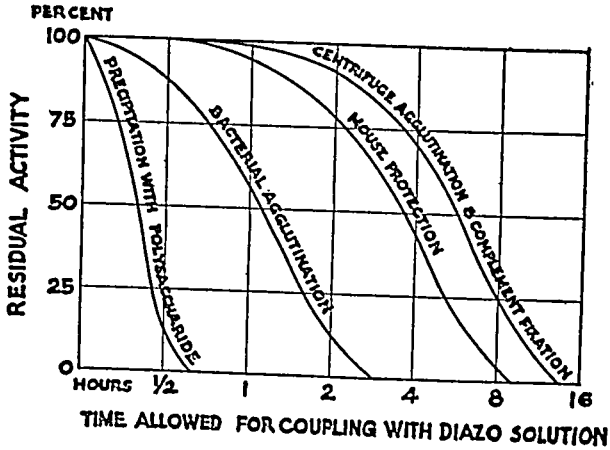

FIG. 1. Diagrammatic representation of the effect of diazo compound on the immunological reactivity of antipneumococcus serum. The order in which protective action, centrifuge agglutination and bacterial complement fixation disappear is not invariable.

tion was even more pronounced than columns 1 and 3 in Table II would indicate. If, after the precipitating activity had been completely destroyed, and the agglutinating titer had been cut to a fraction of its original value, serial dilutions of the serum were incubated with bacteria and the latter then centrifuged, they packed to form a coherent floccule of agglutinated bacteria even in serum dilutions which would otherwise have shown no agglutination. In some experiments (*e.g.* Experiment 2, in Table II) the difference between the apparent agglutinating titer and the centrifuge agglutination titer of the coupled serum was striking. The antibody had apparently retained its affinity for the bacteria, but the deposit of coupled antibody

(*b*) NaCl N/7 up to 0.4 cc. and (*c*) 0.02 cc. of a sheep cell lipoid suspension fortified with sterols (Eagle, 1935*b*) were mixed, shaken and incubated for 4 hours at 37°C. The tubes were then centrifuged and aggregation read after the addition of 1 cc. of N/7 NaCl.

Lipoid Complement Fixation.—(*a*) Serially decreasing quantities of treated serum, (*b*) NaCl N/7 up to 0.2 cc., (*c*) 0.2 cc. of the lipoid suspension diluted 1:100 with NaCl and (*d*) 0.2 cc. of a 1:10 guinea pig complement were mixed and placed in the ice box for 4 hours, followed by ½ hour at 37°C. 0.4 cc. of a 1½ per cent suspension of sensitized cells was then added, and the results read after ½ hour at 37°C.

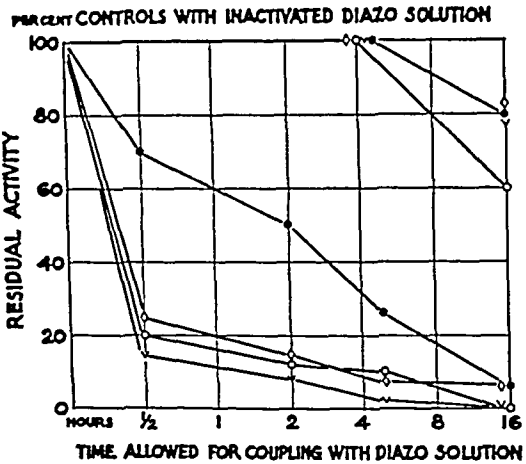

FIG. 2. The effect of diazotized sulfanilic acid on the hemolytic (O — O), hemagglutinating (● — ●), lipoid flocculating (◇ — ◇) and lipoid complement fixing (v — v) activities of an anti-sheep red blood cell serum.

As is seen in Fig. 2, the hemolytic, lipoid-flocculating and lipoid complement-fixing activity of the antiserum fell off exactly in parallel. The hemagglutinating activity, however, seemed somewhat more resistant to the diazo inactivation. The difference in susceptibility was not nearly as pronounced as that found between the carbohydrate-precipitating and bacteria-agglutinating activity of antipneumococcus serum, or between the toxin-combining and toxin-precipitating activity of antidiphtheria serum; nevertheless, it suggests either the presence of several antibodies in an amboceptor serum, or a dissociation between the hemagglutinating and other manifestations of a single antibody.

antibody deposit seems to be less "sticky" than normally. We must leave as an open question whether the addition of the diazo compound to the antibody molecule makes the antibody deposit on the surface of the bacteria more water-soluble, and correspondingly less susceptible to aggregation by electrolyte (Eagle, 1930); or, whether the diazo compound inhibits aggregation by blocking some of the specifically reacting groups in the antibody molecule, which would impair that combination between the antibody deposit and other unsensitized bacteria suggested by Marrack[5] as the cause of bacterial agglutination.

Amboceptor Serum

A rabbit antiserum against sheep red blood cells added to a washed suspension of the same cells causes (*a*) hemagglutination, and, in the presence of complement (*b*) hemolysis. The serum also causes (*c*) the flocculation of lipoids extracted from the cells, and (*d*) gives a positive complement fixation reaction with the lipoid suspension. The titer of the serum with respect to all four reactions was followed quantitatively as amboceptor serum coupled with the various diazo compounds. A single experiment with diazosulfanilic acid is described in Protocol 2 and Fig. 2; similar results were obtained with the other diazo compounds tested.

Protocol 2

10 cc. of a rabbit anti-sheep cell serum were mixed with 5 cc. of N/7 diazotized Na sulfanilate. 3 cc. samples were withdrawn after ½, 2, 4 and 16 hours at room temperature, and the reactions terminated by the addition of 2 cc. N/7 Na sulfanilate. The control consisted of 2 cc. serum plus 3 cc. of a 1:2 diazosulfanilate:sulfanilate mixture, tested after 16 hours at room temperature. The following technic was used to determine the several reaction titers.

Hemolysis.—(*a*) Serially decreasing quantities of treated serum, (*b*) 0.2 cc. of a 3 per cent sheep cell suspension, (*c*) NaCl N/7 up to 0.8 cc. and (*d*) 0.2 cc. of 1:10 guinea pig complement were mixed in the order named. The degree of hemolysis was read after ½ hour at 37°C.

Hemagglutination.—(*a*) Serially decreasing quantities of treated serum, (*b*) NaCl N/7 up to 0.4 cc. and (*c*) 0.05 cc. of a 3 per cent sheep cell suspension were mixed in the order named. Agglutination was read after 2 hours at room temperature and checked after 18 hours in the refrigerator.

Lipoid Flocculation.—(*a*) Serially decreasing quantities of treated serum,

[5] Marrack, page 115.

TABLE IV

Showing That Coupling a Rabbit Antiserum vs. Horse Serum with Diazosulfanilic Acid Destroys the Affinity of the Antibody for the Corresponding Antigen. (All Coupling Overnight at Room Temperature)

Serum	N/7 diazo solution	N/7Na sulfanilate added after overnight coupling	Precipitating activity of the treated serum																			Conclusion
			1 Cc. serum + N/7 NaCl up to 1.6 cc. + 0.1 cc. of 1:50 horse serum							2 0.8 cc. of supernatant fluids of (1) + 0.1 cc. of 1:50 horse serum. (Test for residual free antibody)							3 0.4 cc. of supernatant fluids of (1) + 0.2 cc. of fresh antiserum. (Test for residual free antigen)							
										Tube							Tube							
			1.6 cc.	0.8 cc.	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	1	2	3	4	5	6	7	1	2	3	4	5	6	7	
cc.	*cc.*	*cc.*																						
5	0	0	4	4	4	4	4	3	2	+	+	+	+	±	0	0	0	0	0	0	±	+	+	The decreasing precipitation in section 1 is due to the decreased ability of the antibody to combine with antigen, as shown by the presence of free antigen
5	0.25	0.375	4	4	4	4	3	2	1	+	+	+	+	0	0	0	0	0	0	±	+	+	+	
5	0.5	0.75	4	4	4	3	2	1	1	+	+	±	0	0	0	0	0	0	±	+	+	+	+	
5	1.0	1.5	4	3	2	1	1	1	±	0	0	0	0	0	0	0	±	+	+	+	+	+	+	
5	2.0	3.0	1	1	1	±	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+	
5	4.0	6.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+	
5	8.0	14.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+	
5	10 cc. of a 1:1.5 diazosulfanilate: sulfanilate mixture (comparable to row 6)		4	4	4	4	3	2	1	+	+	+	±	0	0	0	0	0	±	+	+	+	+	Control antiserum, treated with inactivated diazo compound, can still combine with antigen, as shown by the absence of free antigen in the first few tubes

Rabbit Antisera vs. Egg Albumen

As with every antibody studied in this paper, coupling this antiserum with the diazo compound resulted in a progressive and eventually complete disappearance of antibody activity, as manifested both by precipitation and complement fixation. The two reactions fell off in parallel.

Rabbit Antiserum vs. Horse Serum

It became of interest to ascertain whether the progressive decrease in the reactivity of precipitating rabbit antisera as they coupled with diazotized sulfanilic acid was due to a loss in combining affinity for antigen, as in the case of horse antipneumococcus sera, or whether combination proceeded normally, and only the secondary aggregation was affected, as with diphtheria antitoxin. The inhibition experiment with rabbit antiserum *vs.* horse serum, summarized in Table IV, seemed to show that the former was the case: the gradual disappearance of precipitating activity was apparently due to the fact that the coupled antibody was losing its affinity for the homologous antigen (*cf.* page 629).

Serum from Syphilitic Patients

The results summarized in Table V are self explanatory. The diazo compound, added to human syphilitic serum in the quantities indicated in the table, caused an extraordinarily rapid and complete disappearance of the characteristic reactivity with alcoholic extracts of beef heart. Both the flocculation and Wassermann reactions, originally positive up to 1:32 dilutions of serum, became completely negative within 1 minute after the addition of 1 volume of N/7 diazo compound to 3 volumes of serum.

Horse and Rabbit Antityphoid Serum

The effect of diazotized sulfanilic acid on the agglutinating activity of horse antityphoid serum is illustrated in Table VI and Fig. 3, in which one of four similar experiments is summarized. There was the usual progressive decrease in antibody activity; but the amount of coupling required to produce this inactivation seemed significantly greater than in the case of the sera previously described. Although only the results with the horse serum are given in Fig. 3, rabbit antiserum was similarly inactivated.

TABLE VI

The Effect of Diazotized Sulfanilic Acid on the Immunological Reactivity of Antityphoid Serum

To 20 cc. of 1:10 serum were added 20 cc. of N/7 sodium diazosulfanilate. At the intervals indicated in the table, 7 cc. samples were withdrawn and the reaction terminated by the addition of 7 cc. N/7 sodium metanilate. The usual controls were set up by adding 2 cc. of 1:10 serum to 6 cc. of (*a*) 0.85 per cent NaCl; (*b*) a 2:1 diazosulfanilate: sulfanilate mixture.

Treatment of serum	Agglutination								Relative cataphoretic mobility of sensitized bacteria at pH						Isoelectric points* of sensitized bacteria
	0.8 cc.	0.2 cc.	0.05 cc.	0.025 cc.	0.0125 cc.	0.0062 cc.	0.0031 cc.	0.0016 cc.	4.7	4.4	4.1	3.8	3.5	3.2	
															pH
Overnight control with N/7 NaCl	4	4	4	4	4	4	2	0	−2.9	+1.5	+3.1				4.5
Overnight control with inactivated diazo solution	4	4	4	4	4	4	2	0	−2.5	0	+2.9				4.4
1 min. coupling	4	4	4	4	4	3	±	0	−3.0	+1.5	+3.3				4.5
4 " "	4	4	4	4	1	0	0	0	−3.3	−0.7	+3.2				4.35
16 " "	4	4	4	4	2	0	0	0	−4.3	−1.8	Barely +	+3.2			4.15
60 " "	4	4	4	3	0	0	0	0			−4.2	−2.6	+2.1	+4.1	3.65
240 " "	4	4	2	2	0	0	0	0				−2.8	−1.0	+1.2	3.35
Overnight "	0	0	0	0	0	0	0	0	No significant cataphoretic mobility						—

* These are the isoelectric points of bacteria sensitized with a fixed amount of coupled antiserum, and therefore with decreasing quantities of effective antibody. However, a preceding experiment had shown that over a 64-fold range in antibody concentration the isoelectric point of these bacteria was practically independent of the degree of sensitization. The values given for the isoelectric point were obtained from the cataphoretic mobilities by graphic interpolation.

Of particular interest was the effect of the diazo compound on the isoelectric point of antibody to *Bact. typhosum*. Mudd and Joffe observed an acid shift of 0.6–0.8 pH units in the isoelectric point of sensitized typhoid bacteria treated with formaldehyde, probably due to a reaction between basic groups in the antibody film and the formaldehyde. *A priori*, one would expect that the addition of a sulfanilate radical in place of or adjacent to an NH, NH_2 or OH group in a molecule of antibody protein would also result in a more acidic

TABLE V

The Effect of Sodium Diazosulfanilate on the Reactivity of Syphilitic Serum

To 9 cc. serum were added 3 cc. N/7 Na diazosulfanilate. At the stated intervals, 1.6 cc. were withdrawn and the reaction terminated by the addition of 0.8 cc. N/7 Na sulfanilate. The usual controls were set up by diluting 2 cc. samples of serum with 2 cc. of salt solution and with 2 cc. of diazosulfanilate solution inactivated by the addition of sulfanilate.

Time allowed for coupling	Flocculation with beef heart lipoid: Cc. serum + 0.85% NaCl to 0.4 cc. + 0.03 cc. flocculation antigen (Eagle, 1932)					Wassermann reaction: Result with varying amounts of serum					
	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.0125 cc.	0.001 cc.
Control with N/7 NaCl after 60 min.	+	+	+	+	±	+	+	+	+	±	0
Control with inactivated diazo solution after 60 min.	+	+	+	+	±	+	+	+	+	±	0
¼ min. coupling	+	±	0	0	0	0	0	0	0	0	0
½ " "	±	0	0	0	0	0	0	0	0	0	0
1 " "	0	0	0	0	0	0	0	0	0	0	0
2 " "	0	0	0	0	0	0	0	0	0	0	0

antibody, and that the greater the number of sulfanilate groups so introduced, the more acid would be the isoelectric point of the coupled protein. The strain of *Bact. typhosum* described by Mudd and Joffe was peculiarly well suited to test this surmise. Most other bacteria and suspended particles have a more or less pronounced cataphoretic potential. In consequence, their isoelectric point after sensitization with antiserum is intermediate between that of the antibody deposit and that of the uncovered bacterial surface, the

In this particular experiment, the most acid isoelectric point observed was pH 3.35; in another experiment, an antibody was obtained with an isoelectric point at pH 2.7; and, as is indicated by the dotted line in Fig. 3, there is reason to believe that on further coupling, just before the antiserum loses all of its reactivity, the residual traces of antibody may have an isoelectric point even more acidic than pH 2.7.

DISCUSSION

1. Every antibody studied in this paper lost all reactivity with the corresponding antigen on sufficient coupling with the diazo compounds. The susceptibility of the several antibodies to this inactivation varied widely, from the almost instantaneous destruction of syphilitic reagin and the rapid inactivation of a rabbit antiserum against egg albumen, to the slow decrease in the agglutinating activity on an antityphoid serum.

2. The gradual and eventually complete disappearance of antibody activity as the sera coupled with the diazosulfanilic acid might be due to the inactivation of an increasing proportion of antibody molecules; however, it might also reflect a progressive decrease in the reactivity of each antibody molecule. To take a specific example: if the agglutinating titer of a treated serum was found to be only 25 per cent of its original value, this could be interpreted on the basis that 75 per cent of the antibody molecules had been inactivated, leaving 25 per cent with normal activity, or, it might mean that every antibody molecule had lost approximately 75 per cent of its agglutinating activity.

On *a priori* statistical grounds, it seems improbable that at any one moment, some molecules of antibody protein would have reacted with *e.g.* 50 molecules of diazo compound and be completely inactivated, while others would have combined with *e.g.* only 5 molecules of the diazo substance. The experiment with antityphoid serum provided a clear answer to this question as regards this particular antibody. As coupling proceeded, the isoelectric point of the residual active antibody, determined by measuring the cataphoretic mobility of bacteria sensitized with that antibody, became progressively more acidic. This indicates that the residual reactivity was not due to residual normal antibody, but to a chemically altered antibody which

particular value obtained varying to a large extent with the degree of sensitization. This strain has no significant cataphoretic potential against water. When the organism combines with antibody, it does develop a cataphoretic mobility and a cataphoretic isoelectric point which are presumably determined solely by the bound antibody and are largely independent of the degree of sensitization. It was thus possible to follow the progressive change in the isoelectric point of the antibody to *Bact. typhosum* as it coupled with the diazo compound, and to correlate that change with the progressive decrease in its im-

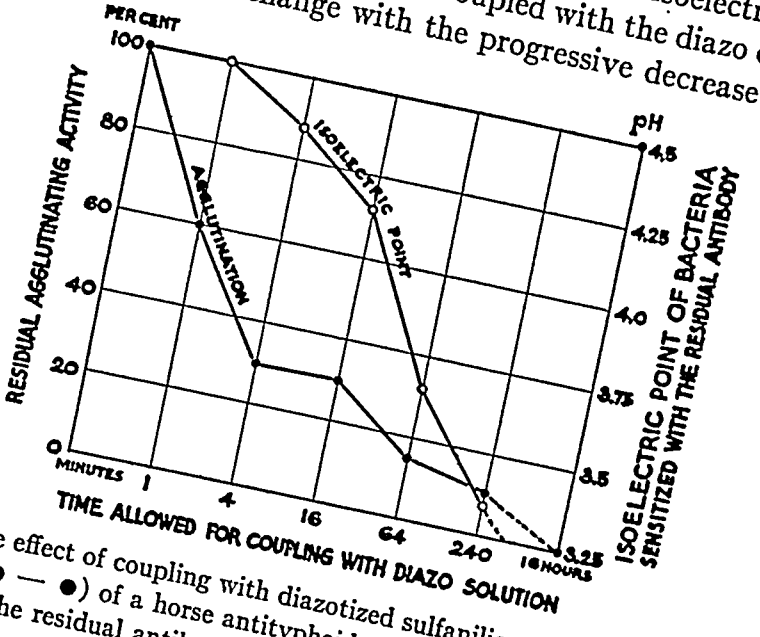

FIG. 3. The effect of coupling with diazotized sulfanilic acid on the agglutinating activity (● — ●) of a horse antityphoid serum, and on the isoelectric point (○ — ○) of the residual antibody.

munological reactivity. Aliquot samples were periodically with drawn from a serum:diazo mixture, and the reaction terminated b the addition of sulfanilic acid. The residual agglutinating titer was obtained in the usual manner; and the cataphoretic isoelectric point of bacteria sensitized with that sample and washed free of serum was then measured, using acetate buffers for pH values between 5 and 3.5 and dilute HCl for the more acid ranges. The results are summarized in Fig. 3, and are self explanatory. As the antibody coupled with the diazosulfanilate, there was a progressive acid shift in the isoelectric point of the antibody as measured on the surface of bacteria, paralleling the progressive decrease in the antibody titer of the serum.

NH_2 groups, the imidazole ring of histidine, the NH of proline and hydroxyproline, the indole group of tryptophane and the phenyl group of tyrosine. The fact that every antibody studied in this paper was eventually completely destroyed by the diazo compound would seem to imply that one or all of these groups are essential for the reaction with antigen.[6] The present series of experiments do not allow of a more accurate localization, which must await the development of a technic whereby individual groups on protein can be blocked or modified.[7]

5. It was shown for two antibodies, rabbit antiserum *vs.* horse serum, and horse antiserum *vs.* type-specific pneumococcus polysaccharide, that the complement-fixing and precipitating titer of the serum after coupling with diazosulfanilic acid apparently depended solely on the ability of the serum to combine with antigen; these manifestations of antibody activity fell off together, and in parallel with the decreased combining affinity of the antibody for antigen. This suggests, with the reservation noted on page 629, that so long as these two antibodies remained capable of combining with antigen, the secondary objective manifestations of this combination followed as a matter of course, unaffected by the coupling with the diazo compound; and that the groups affected by the latter are not primarily concerned in the secondary aggregation or complement fixation.

In direct contrast, diphtheria antitoxin completely lost its ability to give flocculation with antigen on moderate coupling with diazo compound, while it still retained almost its original combining af-

[6] It is conceivable, although improbable, that the observed loss in immunological reactivity might be due to steric hindrance: the diazo molecules, added on to the antibody protein, might block some specifically reacting group in the latter adjacent to, but not identical with, the group which binds the diazo compound.

[7] Although not germane to the present paper, it is to be noted that diazo compounds inactivate Type I pneumococcus polysaccharide, but have no effect on the Type II polysaccharide. The difference may well be due to the fact that the Type I polysaccharide contains NH_2 groups capable of reacting with the diazo compound; while the Type II derivative, lacking NH_2 groups, may not react with the diazo compound. If this proves to be correct, it indicates that not only the COOH groups (Chow and Goebel), but the NH_2 groups as well may be essential for the immunological reactivity of the Type I polysaccharide.

became progressively more acidic and less reactive as it added on more and more of the diazotized sulfanilate. The possibility of non-specific adsorption of coupled acidic protein by the bacteria was excluded by the fact that when the serum had lost all agglutinating activity it had no effect on the isoelectric point of the bacteria. There is no reason to doubt that a similar situation obtains in the case of all the antibodies studied in this paper: that each molecule combines with progressively increasing quantities of the diazo compound, gradually losing its immunological reactivity as it does so. Indeed, the experiments with antipneumococcus serum and with diphtheria antitoxin furnish further evidence that such is the case. Partial coupling of the diphtheria antitoxin molecule completely inhibited the flocculation reaction with toxin without impairing its ability to combine with and neutralize the toxin. Moreover, the gradual disappearance of flocculating activity was not associated with a corresponding decrease in the flocculating titer of the serum: the optimum proportion of serum and toxin for flocculation remained the same, only the degree and velocity of the flocculation being affected. This strongly suggests that all the antibody molecules were being simultaneously affected. In the case of antipneumococcus serum, the first change noted was a disappearance of ability to react with carbohydrate, and the serum became almost wholly inactive in this respect before there was any decrease in its agglutinating action. It is difficult to explain these several observations with antityphoid, antipneumococcus and antitoxin sera on any basis other than the progressive addition of more and more diazo molecules to each molecule of antibody, the immunological reactivity being progressively modified as coupling proceeded.

3. The fact that four other diazo compounds as different as diazotized arsanilic, *p*-aminobenzoic, *p*-amino-2-naphthol-4-sulfonic and 4-amino-2-sulfonic acids had qualitatively the same effect as diazosulfanilic acid on antipneumococcus serum, amboceptor serum and diphtheria antitoxin indicates that the observed effects were largely independent of the type of substance added on to the antibody molecule.

4. As was shown in a preceding paper, it is probable that diazo compounds react with at least six groups in proteins: free aliphatic

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concentration, etc., exemplified by the data summarized in Table VII, suggests that antibodies differ in the number and type of specifically reacting groups. It should be pointed out that the relative times indicated in Table VII are not a direct measure of the vulnerability of the antibody, in view of the progressive spontaneous deterioration of the diazo compound.

TABLE VII

The Varying Susceptibility of Different Antibodies to Inactivation by M/7 Na Diazosulfanilate

Antiserum	Type of reaction	Approximate time (in hours) required for 75 per cent inactivation				
		Diazo: serum = 1:6	Diazo: serum = 1:4	Diazo: serum = 1:2	Diazo: serum = 1:1	Diazo: serum = 10:1
Syphilis reagin	Wassermann and flocculation			0.01		
Diphtheria antitoxin	Ramon flocculation		0.05–0.3	0.02		
	Animal protection		4–24	0.25		
Antipneumococcus horse serum	Carbohydrate precipitation	1.5		0.2 –0.07	0.02–0.06	
	Agglutination	24		0.24–1	4	
	Mouse protection			0.5 –2	1	
Rabbit antiserum *vs.* horse serum	Precipitation				0.25	
Horse antityphoid serum	Agglutination			24	8–24	0.5–4

SUMMARY

Sufficient coupling with any of five different diazo compounds eventually destroyed the reactivity of all the antisera here studied. The rates of inactivation varied considerably among the several antisera. By stopping the reaction at intervals, it was possible to prepare partially inactivated antibodies of peculiarly modified reactivity.

Thus, the flocculating activity of diphtheria antitoxin with toxin was completely destroyed long before there was any demonstrable impairment of its protective titer *in vivo*. The first change induced in

finity for the antigen. In the case of this antibody, the first groups to be attacked by the diazo compound (and there is as yet no indication as to which of the six groups are most reactive) apparently play no part in the combination of its antitoxin with toxin, but are essential for the flocculation of the formed compound.

Similarly, the first few groups in the proteins of an antipneumococcus serum to react with diazo compound seem essential for its precipitating reactivity with the type-specific capsular carbohydrate, but play little or no part in its reaction with the whole bacterial cell. On further coupling with the diazo compound, antibody groups are inactivated which seem essential for the agglutination of bacteria but which are not the actual combining groups. The chemical identification and differentiation of the groups responsible for precipitation with polysaccharide, for bacterial agglutination and for combination with bacteria await the development of an appropriate chemical technic.[7]

The recent work of Chow and Goebel, who found that formaldehyde caused a reversible inactivation of the precipitin to Type I pneumococcus polysaccharide, suggests that free amino groups are essential for the precipitating reactivity of this particular antibody. It will be of interest to ascertain whether formaldehyde causes a dissociation between the agglutinating and precipitating activity of a pneumococcus antiserum analogous to that produced by diazo compounds.

6. It has been suggested (Heidelberger; Marrack) that the precipitation of antigen-antibody compounds is not due to an altered solubility of the combined antibody as suggested by Eagle (1930), but results from the combination of antigen-antibody aggregates with similar aggregates by virtue of specifically reacting groups on the surface of each, until the complex becomes sufficiently large to become macroscopically visible as sedimenting clumps. The observation that diphtheria antitoxin can be chemically altered so that it remains capable of combining with toxin, but no longer gives the flocculation reaction is difficult to reconcile with the latter hypothesis.

7. The extraordinary difference in the rates of inactivation of the various antibodies under comparable conditions of volume, diazo

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antipneumococcus horse sera was the apparently complete loss of reactivity with the capsular carbohydrate at a time when the agglutinating, animal-protecting and complement-fixing activity of the sera were only slightly affected. On further coupling, the sera no longer caused visible agglutination; but aggregation of the serum-treated bacteria could be induced by centrifugation. Still further coupling destroyed all antibody activity.

Rabbit antisera to egg albumen and horse serum no longer precipitated the homologous antigen after treatment with diazo compounds, probably due to their failure to combine with the antigen. The hemolytic, complement-fixing and lipoid-flocculating activity of coupled rabbit antisera to sheep red blood cells fell off in parallel; the hemagglutinin seemed somewhat more resistant. The reagin of syphilitic serum was destroyed almost instantaneously by comparatively small amounts of diazo compounds. Finally, in the case of antityphoid agglutinin, the isoelectric point of the coupled antibody, measured on the surface of specifically sensitized bacteria, was found to shift from an original value of pH 4.7 to one of less than pH 2.7 as progressively more sulfanilic acid radicals added on to the antibody molecule.

The groups in protein which participate in its reaction with diazo compounds probably include aliphatic amines, the imidazole ring of histidine, the indole group of tryptophane, the NH of proline and hydroxyproline and the phenyl group of tyrosine. Although it has been possible to modify antibodies chemically so that they combine with the corresponding antigens without causing their aggregation, the experiments here described furnish no indication as to which of these groups in antibody protein are primarily concerned in the antigen-antibody reaction, and which are responsible for the secondary flocculation. Such localization awaits the development of a technic for attacking individual groups in the protein molecule.

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NEUTRALIZATION TESTS WITH SERA OF CONVALESCENT OR IMMUNIZED ANIMALS AND THE VIRUSES OF SWINE AND HUMAN INFLUENZA

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Smith, Andrewes, and Laidlaw (1, 2) have noted that the immunity which follows infection of ferrets with either swine or human influenza virus confers a considerable active resistance against the other virus. They further observed, in neutralization tests done in ferrets, that, while each strain was neutralized by admixture with homologous ferret immune serum, neutralization by the heterologous serum was inconstant. In addition, Shope (3) has reported that mice immune to either the swine or human influenza virus resist later infection with the heterologous strain. On the other hand, Francis (4) was unable to demonstrate neutralizing antibodies against the P. R. 8 strain of the human influenza virus in the serum of swine immune to swine influenza. These facts suggest that the active immunity which develops in animals, following infection with either the swine or human influenza virus, is effective against both viruses. Nevertheless, it seems certain that the sera of such animals, although uniformly neutralizing the homologous virus, may or may not neutralize the heterologous virus.

The present experiments were carried out in an effort to determine the factors involved in the development of heterologous virus-neutralizing antibodies following infection or immunization with the swine or human influenza virus. It was hoped that the information obtained would be useful in interpreting the results of experiments in which samples of human serum were tested in duplicate for their ability to neutralize the human and swine influenza viruses (5, 6).

As shown by the data in Table I, the human and the swine influenza viruses were consistently neutralized by their homologous immune sera. Convalescent serum from animals submitted to but a single virus exposure appeared to be as efficient in this respect as that from animals submitted to repeated virus insults. On the other hand, after repeated inoculations of one virus, the serum of the animal was frequently found to exert some effect against the heterologous virus. This was more marked in the cases of animals receiving human influenza virus than in those receiving swine virus. The conditions involved are not strictly comparable, however, because multiple inoculations with the human virus were made intranasally in most instances, whereas the swine virus was frequently administered by other routes.

That the sera from animals immune to the swine influenza virus exerted in certain instances some protection against the human influenza virus is evidenced by the fact that mice receiving mixtures of swine influenza immune serum and human influenza virus developed less extensive pulmonary lesions than their controls. These differences were in some instances so slight as to be of doubtful significance, but in other cases there was undoubted partial protection. The degree of cross-protection could not be positively correlated with the number of exposures of the serum donor to swine influenza virus, although in the only instance in which cross-neutralization by swine influenza immune serum was complete, the mice furnishing the serum had undergone repeated inoculations with swine influenza virus. The results show that sera from animals subjected to repeated inoculations with swine influenza virus are frequently capable of partially neutralizing the P. R. 8 strain of human influenza virus in the amounts employed. By decreasing the amount of virus in the mixtures a dilution might have been reached at which such sera would afford complete protection. It seemed best, however, to have each mixture contain an amount of virus sufficient to kill all or most of the control mice, in order to simplify interpretation of the results obtained.

The sera of ferrets or swine merely convalescent from infection with the human influenza virus exerted little, if any, protection against the swine influenza virus. Mice inoculated with mixtures of such sera and swine influenza virus usually died, just as did their controls. The few that survived the 6 day period of observation exhibited extensive

Materials and Methods

Strains of Virus.—The viruses employed in the present experiments were the P.R.8 strain human influenza virus isolated by Francis in 1934 (7) and the strain 15 swine influenza virus obtained by Shope in Iowa in 1930. Both were well adapted to mice and killed them quite regularly in less than 6 days following infection.

Sera.—The 2 horse sera used were prepared in England (8) and obtained through the courtesy of Drs. Laidlaw, Smith, Andrewes, and Dunkin. All other sera were from animals studied in our laboratories. The ferrets infected with the Alaska strain and the swine infected with the P.R.8 strain were employed in experiments the details of which have not yet been published.

Neutralization Tests.—The tests with swine influenza virus were performed as follows:

Weighed amounts of glycerolated infected mouse lung were ground with sand and suspended in physiological saline to form a 2 per cent suspension. The suspension was allowed to sediment for 10 minutes, and at the end of this time the supernatant fluid was removed by pipette and used as the source of virus. Equal parts of the serum to be tested and the virus were mixed and stored for 2 hours in the refrigerator (4°C.) prior to administration intranasally to the test mice. The mice were lightly etherized and their noses and mouths were then immersed in the serum-virus mixture contained in one side of a slightly tilted Petri dish, as described in a previous paper (3). 4 mice were employed for each test in most instances. On the 6th day after inoculation, at which time the control mice were either dead or very ill, the remaining mice were killed with chloroform and their lungs removed. The neutralizing effect of a serum of unknown potency upon the virus was measured by comparing the extent of the pulmonary lesions in mice receiving a mixture of that serum and virus with the lesions in control mice receiving the virus and normal serum.

The procedure employed in tests made with human influenza virus differed in certain particulars from that outlined above. A centrifugalized 10 per cent suspension of infected mouse lung was used; the serum-virus mixtures were incubated at 37°C. for 30 minutes; and 0.03 cc. of the mixture was given to each mouse. The procedure is described in detail in the following paper (5). Although the exact procedures differed in the tests with swine virus and human virus, nevertheless, a sufficient number of tests with the same virus and the same serum were done in duplicate by the two methods to indicate that the results obtained are closely comparable.

Results of the Neutralization Tests

The results of experiments in which sera from animals immune to either the human or swine influenza virus were tested for their ability to neutralize the two viruses are outlined in Table I.

Swine	1931–32	Pooled sera from hogs convalescent from swine influenza*	1 or more IN	0	0	0	—	4	4	2	2
	15-30	Vaccinated against swine influenza	2-IM	0	0	0	0	4	4	4	—
	15-61	" " " "	2-IM	0	0	0	1	4	4	3	—
	10-77	Convalescent from infection with swine influenza virus alone	1-IN	0	0	0	0	4	4	4	—
	13-56	Convalescent from swine influenza	1-IN	0	0	0	0	4	3	4	4
	11-47	Vaccinated and immune to swine influenza	3-IM, 1-IN	0	0	0	0	3	4	3	4
	14-44	Convalescent from swine influenza	2-IN	0	0	0	0	2	3	3	3
Rabbits	13-19	Vaccinated with swine influenza virus	2-IP	0	0	0	0	4	4	3	4
	13-20	" " " " "	2-IP	0	0	0	0	4	4	4	4
Horse	I.H. 4	Hyperimmunized against swine influenza virus (Laidlaw *et al.*)	6-SC	0	0	0	0	3	2	2	3
		(*b*) *Animals previously vaccinated or infected with human influenza virus*									
Mice	Lot 4	Vaccinated and immune to P.R.8 human influenza virus	1-SC, 1-IP, 1-IN	4	4	3	2	0	0	0	0
	" 1	" " " " " " " "	1-SC, 1-IP, 3-IN	0	0	0	0	0	0	0	0
	" 2	" " " " Phila. " " "	1-SC, 1-IP, 2-IN	0	0	0	0	0	0	0	0
	" 3	" " " " " " " "	2-IP, 3-IN	0	1	0	1	0	0	0	0

IP = intraperitoneally.
IN = intranasally.
SC = subcutaneously.
IM = intramuscularly.
0 = mouse with no detectable influenzal lung lesions postmortem.
1 = mouse with influenzal pneumonia involving up to ¼ of lung at postmortem.
2 = " " " " " from ¼ to ½ " " "
3 = " " " " " " ½ " ¾ " " "
4 = " " " " " " ¾ to all of lung at postmortem. Mice showing lung lesions to this extent usually died.

* The term swine influenza indicates that both swine influenza virus and *H. influenzae suis* were used for infection.

TABLE I

Animal No.	Animals from which serum was obtained	No. and route of inoculations	Serum tested against: Swine influenza virus, strain 15 — Extent of pulmonary lesions in Mouse No.				Serum tested against: Human influenza virus, strain P.R. 8 — Extent of pulmonary lesions in Mouse No.			
			I	II	III	IV	I	II	III	IV
	(a) Animals previously vaccinated or infected with swine influenza virus									
Mice Lot A	Vaccinated and immune to swine influenza virus	2–IP, 1–IN	0	0	0	0	4	4	4	4
" B	Convalescent from swine influenza virus infection	1–IN	0	0	0	0	4	4	4	4
" C	Vaccinated and actively immune to swine influenza virus	1–SC, 1–IN	0	0	0	0	2	3	2	3
" D	" " " " " " "	3–IP or SC, 1–IN	0	0	0	0	3	3	4	4
" E	" " " " " " "	3–SC, 1–IN	0	0	0	0	4	4	4	4
" F	" " " " " " "	3–IP, 1–IN	0	0	0	0	4	4	4	3
" G	" " " " " " "	3–SC, 1–IN	0	0	0	0	3	3	2	2
" H	" " " " " " "	3–IP, 1–IN	0	0	0	0	1	3	1	2
" I	" " " " " " "	3–SC, 1–IN	0	0	0	0	4	3	4	—
" J	" " " " " " "	3–IP, 1–IN	0	0	0	0	3	3	2	2
" K	" " " " " " "	3–SC, 1–IN	0	0	0	0	4	3	3	3
" L	" " " " " " "	3–IP, 1–IN	0	0	0	0	3	1	2	2
" M	" " " " " " "	1–SC, 1–IN	0	0	0	0	1	2	2	2
" N	" " " " " " "	1–IP, 1–IN	0	0	0	0	2	0	2	0
" O	" " " " " " "	2–IP, 1–IN	0	0	0	0	0	4	1	—
Ferrets 5-5, 5-7, 5-8, 6-0	" " " " " " "	1 or 2 SC, 1–IN	0	0	0	0	4	4	0	—

		(c) Animals previously infected with both swine and human influenza virus									
Swine	16-45	Normal		4	4	4	4	4	4	4	4
	"	Convalescent from P.R.8 virus infection (19 days)	1-IN	4	4	4	1	0	0	0	1
	"	31 days after P.R.8 virus IN, 11 days after swine influenza virus	1-IN	0	0	0	0	0	0	0	0
	16-57	Normal		4	4	4	4	4	4	4	4
	"	Convalescent from P.R.8 virus infection (19 days)	1-IN	4	4	4	4	0	0	0	0
	"	11 days after 2nd P.R.8 virus inoculation, 31 days after P.R.8 virus	2-IN	4	4	4	4	0	0	0	0
	"	11 days after swine influenza virus	1-IN	0	0	0	0	0	0	0	0
	16-59	Normal		4	4	4	4	4	4	4	4
	"	Convalescent from P.R.8 virus infection (19 days)	1-IN	4	4	4	—	0	0	0	0
	"	19 days after 2nd P.R.8 virus inoculation	2-IN	3	4	4	4	0	0	0	0
	"	After inoculation of swine influenza virus	1-IN	0	0	0	0	0	0	0	0
		(d) Control animals									
Mice		Normal		4	4	4	4	4	4	4	4
Ferret		"		4	4	4	4	4	4	4	4
Swine		"		4	4	4	4	4	4	4	4
Rabbit		"		4	4	4	4	4	4	4	4
Horse		"		4	4	4	4	4	4	4	4

TABLE I—*Concluded*

Animal No.	Animals from which serum was obtained	No. and route of inoculations	Serum tested against: Swine influenza virus, strain 15 — Extent of pulmonary lesions in Mouse No.				Serum tested against: Human influenza virus, strain P. R. 8 — Extent of pulmonary lesions in Mouse No.			
			I	II	III	IV	I	II	III	IV
	(*b*) *Animals previously vaccinated or infected with human influenza virus*									
Ferrets 3-00	Alaska strain human influenza virus	1–IN	4	4	3	3	0	0	0	0
3-26	" " " " "	2–IN	4	4	4	4	0	0	0	0
"	" " " " "	2–IN, 1–SC	4	4	4	4	0	0	0	0
2-29	" " " " "	1–IN	4	4	4	4	0	0	0	0
2-78	Phila. " " " "	2–IN	4	4	4	4	0	0	0	0
3-12	" " " " "	1–IN	4	4	4	4	0	0	0	0
"	Alaska " " " "	4–IN, 1–SC	4	4	4	—	0	0	0	0
3-25	" " " " "	2–IN	4	2	1	3	0	0	0	0
"	" " " " "	3–IN, 1–SC	4	4	4	4	0	0	0	0
2-15	" " " " "	3–IN	4	2	3	3	0	0	0	0
1-44	Phila. " " " "	3–IN, 1–SC	2	1	1	1	0	0	0	0
1-41	P.R.8 " " " "	4–IN	0	0	0	0	0	0	0	0
1-76	" " " " "	4–IN	0	1	1	—	0	0	0	0
1-14	" " " " "	5–IN, 1–SC	0	0	0	0	0	0	0	0
Swine 16-18	" " " " "	Contact infection	0	0	2	0	0	0	0	0
16-15	Exposure to swine infected with P.R.8 strain human influenza virus	1–IN	4	4	4	4	0	0	1	0
15-99	Convalescent from human influenza virus infection, P.R.8 strain	1–IN	4	4	4	4	0	0	0	0
16-05	Same as above	1–IN	4	4	3	3	0	0	0	0
Rabbit 1-29	" " "	2–IP	4	4	3	0	0	0	0	0
Horse I.H. 2	Vaccinated with human influenza virus P.R.8	20–SC	0	0	0	0	0	0	0	0
	Hyperimmunized against W.S. strain human influenza virus (Laidlaw *et al.*)		0	0	1	0	0	0	0	0

DISCUSSION

The findings presented indicate that certain antigenic components are possessed in common by the human and swine influenza viruses. They also suggest that the common antigen is present in a more active concentration in the human than in the swine virus.

It seems likely that the virus neutralization test is of sufficient accuracy to indicate the nature of the virus involved in earlier influenzal infections of man or animals. The tendency of repeated exposures to virus to diminish the specificity of the reaction would constitute the main source of error. Complete neutralization of both human and swine influenza virus by an unknown sample of serum might mean that its donor had undergone earlier infections with both viruses, or that he had suffered repeated exposures to one or the other virus. Complete neutralization of one virus with no neutralization, or only partial neutralization, of the other would probably indicate, however, the character of the earlier infection.

SUMMARY

Human and swine influenza viruses were regularly neutralized by their homologous immune sera. However, the sera of animals convalescent from infection with either the swine or human influenza virus possessed little, if any, neutralizing capacity for the heterologous virus. Hyperimmunization of animals against swine influenza virus tended to increase the neutralizing capacity of their sera for human influenza virus, but in an inconstant fashion, whereas repeated inoculations with human influenza virus frequently resulted in sera with strong neutralizing activities against swine influenza virus. These observations serve to emphasize both the immunological distinctiveness and the interrelationships of swine and human influenza viruses.

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influenzal pulmonary lesions at autopsy. However, the sera from ferrets and mice that had undergone repeated exposures to human influenza virus tended to neutralize the swine influenza virus and the degree of this heterologous neutralization corresponded roughly with the number of virus exposures undergone by the serum donor. It appeared from this that, while the virus-neutralizing properties of sera of animals convalescent from human influenza virus infection were specific, hyperimmunization tended to broaden the range of activity of these sera so that they finally acquired the ability to neutralize the heterologous as well as the homologous virus. Thus the sera of the repeatedly inoculated ferrets, 1-44 and 1-76, completely neutralized an amount of swine influenza virus that proved fatal for all the control mice, while sera from ferrets 2-29 and 3-00, receiving only a single inoculation of human influenza virus, were devoid of protective power against the same amounts of swine influenza virus. Furthermore, in the case of ferrets 3-12 and 3-25, the neutralizing capacity of the serum against swine influenza virus increased during the course of successive reinoculations with human influenza virus. The serum of mice (lots 1, 2, 3) vaccinated with human influenza virus and subjected thereafter to repeated intranasal inoculations with the human virus, also protected completely against the swine virus. Similarly, the sera of animals of two non-susceptible species (rabbit 1-29 and horse I. H. 2), immunized against the human influenza virus, neutralized the heterologous virus, whereas the serum of animals of the same species (rabbits 13-19–20 and horse I. H. 4), immunized against swine influenza virus, protected but little against the human influenza virus.

The specificity of convalescent serum for the homologous virus is well demonstrated in the case of swine 16-45, 16-57, and 16-59, which, as a result of a primary infection with human influenza virus, developed antibodies effective only against the human strain. When, subsequently, the swine influenza virus was used for reinoculation of the animals, a specific antibody response to swine virus occurred and the serum then neutralized both the human and swine strains of influenza virus. Repeated inoculation of ferrets or mice with human influenza virus does, however, result in the formation of antibodies effective against the swine influenza virus. It should be noted in this connection that ferrets receiving multiple intranasal inoculations with virus exhibited evidence of illness only following the initial inoculation.

THE INCIDENCE OF NEUTRALIZING ANTIBODIES FOR HUMAN INFLUENZA VIRUS IN THE SERUM OF HUMAN INDIVIDUALS OF DIFFERENT AGES

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Evidence has been presented which shows that, following experimental infection with the virus of human influenza, animals develop a state of resistance to reinfection, and the sera of such recovered animals manifest a specific power to protect mice against infection with the homologous virus (1–3). Furthermore, the sera of human individuals develop protective properties against human strains of virus following recovery from naturally acquired influenza (3). The antibodies which develop have been found to persist for 6 to 8 months at least.

Since the presence of antibodies against the human influenza virus in the serum appears to be an expression of a previous infection with the virus, it is important to determine the frequency with which these antibodies occur in the serum of human individuals in the population at large. To this end, serum was obtained from 136 human beings in New York, Philadelphia, Princeton, and Baltimore, and the sera were tested for their capacity to protect mice against a strain of human influenza virus (P. R. 8). So far as possible, histories concerning previous attacks of influenza were also obtained from the same individuals.

The present report deals with the results of mouse protection tests made with these sera against a strain of human influenza virus (P. R. 8). The individuals from whom the sera were obtained ranged from newborn infants to adults in the eighth decade of life.

Methods and Materials

Sera.—The samples of serum used in these tests were derived from clotted venous blood. The majority of the children's sera were obtained from children

With each series of tests, control tests were made, using one sample of human serum which did not protect mice, and another which afforded complete protection to mice. In all instances the results with the control sera were negative and positive, respectively.

While certain variations in the extent of the pulmonary lesions obtained with the same serum in different tests were observed, they were usually of a minor nature, and were never of sufficient degree to require a complete reclassification of the serum. With the strong concentration of virus used, a 10 to 20 per cent variation in the concentration of virus in different tests might occur without seriously altering the results. Another source of variation which was impossible to control was the size of the mice. So far as possible, mice 4 to 6 weeks of age were used. However, considerable differences in the size of animals occurred, and it has been recognized that large mice are more resistant to infection than small ones of 12 to 15 gm.

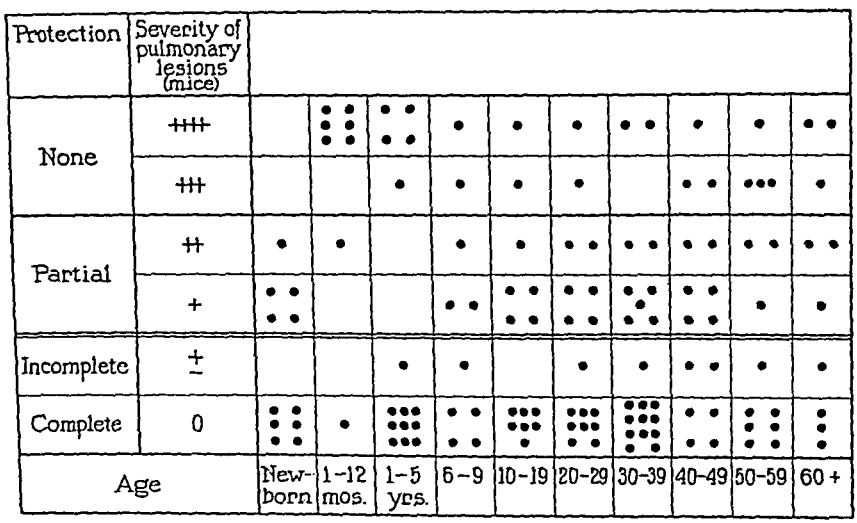

CHART 1. The results of mouse protection tests made with human serum classified according to age of donor. Human influenza virus (P.R. 8).

RESULTS

The results are presented in Chart 1, in which each black circle represents the test made with the serum of a single individual, and the results are distributed according to the age of the donor from whom the serum was obtained. The number of individuals in the different age groups varies, with a relative preponderance of children below 5 years of age, and a disproportionately small number between the ages of

in the wards of the Hospital for the Ruptured and Crippled, New York City.[1] In practically all instances the children were hospitalized because of orthopedic disabilities.

Most of the specimens of serum of elderly individuals were obtained from healthy persons at the Baltimore City Hospital, Baltimore.

Serum was obtained from 18 individuals of various ages in Philadelphia.[2] A few specimens were obtained by Dr. Shope at Princeton.

The majority of the other samples were obtained from staff members or attendants at The Rockefeller Institute.

Virus.—The strain of virus employed in these tests was recovered from an epidemic of influenza in Puerto Rico (4), and has been maintained in white mice through serial passages by the inoculation of suspensions of infected mouse lungs into the nasal passages of normal mice.

Mouse Protection Tests.—The capacity of serum to protect white mice from the action of the virus was tested in the following manner.

Mice infected with virus were killed when moribund, and their lungs removed with aseptic precautions. Weighed amounts of the infected mouse lung were ground with alundum and suspended in physiological salt solution to form a 10 per cent suspension. The suspension was centrifugalized for 10 to 15 minutes at 1500 R.P.M. The supernatant liquid was removed and 0.3 cc. of the fluid was mixed with 0.3 cc. of the serum to be tested. The serum-virus mixture was incubated at 37°C. for 30 minutes. Each of 4 Swiss mice was then inoculated intranasally, while anesthetized with ether, with 0.03 cc. of the mixture.

The mice were observed for 6 days after inoculation. All animals which died during this period were autopsied, and their lungs examined. On the 6th day, all survivors were killed and their lungs were examined for gross pulmonary lesions. The severity of the pulmonary lesions in each mouse was graded by the amount of pulmonary tissue involved as viewed by the naked eye. The various degrees of involvement varied from ±, in which only small pinpoint areas of congestion were seen, to ++++, in which all five lobes of the mouse lung were completely involved. The result of the test with a given serum was determined by making an approximate average of the extent of the lesions in the 4 mice inoculated with each serum-virus mixture. For example, if the lesions in 4 mice were +, +, +, ++, respectively, the final result would be considered +. If the lesions were ++, +++, +, ++, respectively, the result would be classified as ++. If +, +, 0, 0, the result would be considered incomplete protection and graded ±; but for practical purposes these latter results may be considered to indicate complete protection. Mice presenting average lesions of +++ or greater were considered not to have been protected, while in those tests in which an average of +, or ++, lesions appeared, the serum was considered to have afforded partial protection to the mice. In the absence of pulmonary lesions, the result was accounted complete protection.

[1] Through the courtesy of Dr. Stefanson.

[2] Through the kindness of Dr. Joseph E. Stokes, Jr., and Dr. Stuart Mudd.

The results have also been classified according to whether or not the individuals from whom serum was obtained had experienced an attack of influenza (Table II). Tests with the sera of children below the age of 12 years were not included because of the frequent lack of satisfactory histories. In spite of the inaccuracies which occur, a definite

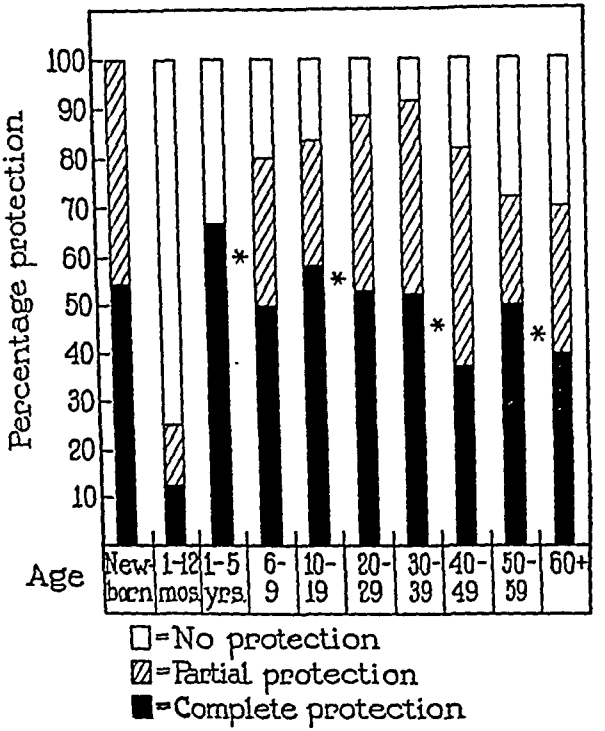

CHART 2. The percentage of human sera exerting protective action against human influenza virus (P.R. 8).

* Indicates per cent of completely protective sera when the two adjacent age groups are combined.

statement regarding previous influenza was obtained from 78 individuals. In this group there appears to be some relation between a history of influenza and a strongly protective serum, since 55.5 per cent of the individuals who were said to have had influenza, and only 39.3 per cent of those without histories of influenza, possessed sufficient circulating antibody to furnish complete protection to mice.

10 and 19. While the entire group is small from a statistical point of view, the trend of the results is sufficiently regular to appear significant.

The sera have been classified, as affording complete protection, partial protection, or no protection. Complete protection was effected by 49 per cent of all sera, partial protection by 29 per cent, and 21 per cent were considered to be non-protective. Distinct differences were noted in the percentage of protective sera at different ages. These figures are presented in Table I and Chart 2.

TABLE I

The Protective Action of Sera of Individuals of Different Ages against Human Influenza Virus

Age of donor	No. of sera	Sera affording					
		Complete protection		Partial protection		No protection	
		No.	*per cent*	*No.*	*per cent*	*No.*	*per cent*
Newborn	11	6	54.5	5	45.5	0	0.0
1 mo.–1 yr.	8	1	12.5	1	12.5	6	75.0
1–5 yrs.	15	10	66.6	0	0.0	5	33.3
6–9 "	10	5	50.0	3	30.0	2	20.0
10–19 "	12	7	58.3	3	25.0	2	16.6
20–29 "	17	9	52.9	6	35.3	2	11.8
30–39 "	23	12	52.1	9	39.1	2	8.8
40–49 "	16	6	37.5	7	43.7	3	18.8
50–59 "	14	7	50.0	3	21.4	4	28..
60+ "	10	4	40.0	3	30.0	2	20.0
Total.....	136	67	49.2	40	29.4	29	21.3

The frequency of completely protective sera in newborn infants is approximately the same as in middle aged individuals. After the 1st month of life, a sharp drop in the percentage of positive sera occurs and persists through the 1st year of life. Between the 1st and 5th years of life, however, the percentage of positive results reaches its height, and 66 per cent of the sera in this age group possess the capacity to protect mice completely against the virus. From that time until the 40th year of life, complete protection was nearly or quite as frequent; thereafter the incidence of protective sera declined somewhat. The trend of the findings is more even if the age groups are combined so as to include greater numbers in each group (Chart 2).

seaboard, it has been possible to test the convalescent serum of 4 subjects in Puerto Rico from whom the original virus material was obtained, and the sera of 15 individuals convalescent from the influenza epidemic which occurred in Alaska in May, 1935.[3] Complete protection resulted with 27, or 87 per cent, of these 31 sera, an incidence of positive sera not otherwise encountered. These results indicate clearly that the occurrence of recent influenza in a group of individuals is followed by a high proportion of strongly protective sera.

In addition to the sera which contained sufficient antibody to protect mice completely against the human influenza virus, 29 per cent were found to afford partial protection; the latter may, therefore, be considered protective sera of lower titer than those of the former group. With this granted, the sera of all new-born infants are found to contain protective antibodies. After the 1st year of life the incidence of antibodies increases to its maximum between the 20th and 40th years, when 90 per cent of the sera confer complete or partial protection on mice. After the 40th year a gradual but progressive decline in the percentage of protective sera occurs.

Quantitative Aspects of the Protective Tests.—Certain sera which were tested by the present method were also tested by Andrewes, Laidlaw, and Smith (5). These sera were the ones mentioned above, which were obtained from Alaskan convalescents, and in addition there were 6 specimens of dried serum from Philadelphia.[4] The British workers attempt to measure the antibody content of a serum more accurately by testing the protective action of progressive dilutions of the serum against a filtrate of virus suspension. The results with the unknown serum are then interpreted by direct comparison with corresponding dilutions of a standard hyperimmune horse serum. The serum is given a rating on this basis. Thus, S indicates that the test serum is as potent as the standard; S/5 or S/25 indicates that the unknown serum is 1/5 or 1/25 as strong as the standard.

A comparison of the results obtained when the same sera were tested by the method used in the present study and by the method of the

[3] The Alaskan sera were obtained by Drs. H. Pettit and D. S. Pepper, of the University of Pennsylvania, immediately following the epidemic, and were kindly made available to us by Dr. Mudd.

The serum of the Puerto Rican subjects was obtained by Dr. W. C. Earle, of the International Health Division of the Rockefeller Foundation, to whom we should like to express our appreciation.

[4] Kindly sent to us by Dr. Mudd and Dr. Stokes.

The sera of the individuals giving positive histories of influenza may again be grouped according to the time at which the illness was experienced (Table III). The results show that the sera of individuals alleged to have had influenza in 1918 were equally divided among the protective and non-protective groups, a result not largely different from the average of the entire series which was studied. The individuals who did not clearly remember the time of illness were in the older age groups, and may be considered not to have suffered from influenza recently. On the other hand the incidence of completely protective sera was considerably greater among the 12 individuals in this series who had suffered from influenza in the past few years, the majority of whom were observed by one of us. In addition to the 12 samples included in this series, taken from the population along the eastern

TABLE II

The Relation of Results of Serum-Virus Protection Tests to History of Influenza

History of influenza	No. of sera	Protecting	Not protecting
		per cent	*per cent*
Yes	45	55.5 (25)	44.5 (20)
No	33	39.3 (13)	60.7 (20)
Total.....	78	48.7	51.3

Numbers in parentheses indicate the number of samples included.

TABLE III

The Relation of the Time of Illness to Protective Property of the Serum

Time of illness	No. of sera	Protective	Non-protective
1918–23	18	9	9
1933–35	12	10	2
1918 and 1930–35	6	3	3
Indefinite	11	3	8
1934–35 (Convalescents, Puerto Rico and Alaska)	19	17	2

English workers shows that the correlation is quite good (Tables IV and V). All the sera which the latter workers have assayed S or S/5 have been found by our method to be completely protective. Among the sera which are graded as S/25 by the titration method, the agreement is not so consistent; certain of them have, in our hands, been completely protective while others protect partially or very little. Nevertheless, the results indicate that both methods afforded information indicative of the antibody content of the sera studied. The severity of lung lesions in the mouse which result from the intranasal inoculation of a serum-virus mixture is inversely proportional to the antibody content of the serum.

DISCUSSION

The present report presents the results of tests designed for the demonstration of protective antibodies against a strain of human influenza virus in the serum of 136 individuals of all ages. In addition to the fact that statistically their numbers are small, interpretation of the results is limited by several factors: (*a*) that the sera were collected in three large cities along the Atlantic seaboard; (*b*) that the age distribution of the individuals from whom the sera were collected was not the same in all three cities; (*c*) that the history of influenza is unreliable; and (*d*) that the previous data regarding persistence of immunity following influenza are very inconclusive. Nevertheless, the similarity to the results recently reported by Andrewes, Laidlaw, and Smith (5) with sera of human individuals of different ages in England suggests that the results are significant. Although their tests were made with a different strain (W. S.) of human influenza virus, the percentages of completely protective sera at different age periods found in the present study do not differ greatly from theirs.

For purposes of discussion it may be assumed that, apart from newborn infants, the presence of specific protective antibodies in the serum of an individual is the result of past infection with the virus of human influenza. Evidence for the validity of this assumption has previously been presented, which shows that recovery from influenza in the human individual has been accompanied by the development of circulating antiviral bodies, whereas, in the course of the ordinary common cold or of pneumococcus pneumonia, the development of antibodies

TABLE IV

Comparison of Results Obtained with Dried Serum

Sample No.	Severity of pulmonary lesions in Mouse No. 1	2	3	4	Result	Andrewes' results (5)
C-91	++++	++++*	++++*	+	NP	S/25
C-74	++	+	+	+	PP	S/25
C-109	0	0	+	±	IP	S/25
C-111	0	0	0	0	CP	S
C-82	0	0	0	0	CP	S
C-121	+++	+++	++++	++++	NP	S/25

NP = no protection.
PP = partial protection.
IP = incomplete protection.
CP = complete protection.
0 = no gross pulmonary lesions.
± to ++++ = progressive degrees of pulmonary involvement.
S = test serum equal in potency to standard serum.
S/5 = " " " " " " " diluted 1/5.
S/25 = " " " " " " " " 1/25.
* = mouse died.

TABLE V

Comparison of Results Obtained in Tests Made with Alaskan Sera

Serum No.	Influenza convalescent	Severity of pulmonary lesions in Mouse No. 1	2	3	4	Result	Andrewes' classification (5)
1	22 yrs., 6 wks. ago	0	0	0	+	CP	S—S/5
2	60 " severe, 3 wks. ago	0	0	±	0	CP	S
3	40 " mild, 2 " "	0	0	0	0	CP	S/25
4	16 " " 1 wk. ago	0	0	0	0	CP	S
5	70 " severe, 2 wks. ago	0	0	0	0	CP	S—S/5
6	63 " " 2 " "	+	±	++	++	PP	S/5—S/25
7	70 " " 2 " "	0	0	0	0	CP	S
8	44 " " 2 " "	+	0	0	0	CP	Less than S/25
10	17 " " 1 wk. ago	0	0	0	0	CP	S
11	21 " mild, 2 wks. ago	0	0	0	0	CP	S/5
13	42 " severe, 2 " "	0	±	0	0	CP	S/5—S/25
14	18 " mild, 2 " "	0	0	0	0	CP	S/5
15	19 " " 2 " "	0	0	±	0	CP	S/5
16	25 " " 2 " "	+	+	++	+	PP	Less than S/25
17	19 " severe, 3 " "	0	0	0	0	CP	S—S/5
	19 " mild, 1 wk. ago						

It seems quite probable, therefore, that the individuals from whom the partially protective sera were obtained are those who have suffered from influenza in the past, but whose circulating antibodies have decreased with the passage of time. This interpretation is supported by an observation of Andrewes, Laidlaw, and Smith (5), in which they noted a marked decline in the antibody titer of the serum of an individual within 2 years following the attack.

If the partially protective sera are included with the fully protective sera, the trend of the findings is modified. Under these conditions all newborn infants are found to possess antibodies, most probably of maternal origin. These are lost to a great extent in the 1st year of life, and are superseded by antibodies presumably developed in response to infection. Between the 1st and 5th years the serum either protects completely or not at all, suggesting recent infection. Thereafter, the incidence mounts to the 20 to 40 age period, when 90 per cent of the sera contain measurable amounts of protective antibodies. This peak may be attributed to the increased percentage of partially protective sera during these years. Beyond the 40th year the percentage of negative sera increases. Whether the decline in the percentage of positive results among older persons indicates that these individuals have wholly escaped infection, or whether it indicates a further step in the loss of antibodies previously acquired, is problematic. If, however, as the statistics of the 1918 epidemic indicate (8), the case rate was highest in children and young adults, the high incidence of antibodies in the present age groups between 20 and 40 years of age might be considered to be a result of infection experienced in 1918. The decreased percentage of protective sera in the older age groups corresponds with the decreased incidence of infection in adults in 1918. Regardless of the relation of the present study to the epidemic of 1918–19, the results suggest that the human influenza virus is related etiologically to the disease which has prevailed in the isolated epidemics of recent years.

Shope (9) tested 124 of the sera used in the present study against the virus of swine influenza. Certain distinct differences appear in the distribution of antibodies against swine influenza virus when compared with those against human influenza virus. The chief differences observed are, that against swine influenza virus little or no protective

against the human influenza virus has not been observed (3). Moreover, the distribution of the virus infection is widespread, as evidenced by the fact that in the past 2 years strains of virus have been recovered from epidemics of human influenza in England (1, 7), Puerto Rico, the United States (2), and Alaska (7). In view of these facts it is reasonable to utilize the results of the protection tests in an attempt to interpret the epidemiology of human influenza.

Considering only those sera which contained sufficient antibody to protect mice completely against human influenza virus, it was found that, excluding the 1st year of life, influenza attacks individuals of all ages. 59 per cent of those between 1 and 20 years of age, 52.5 per cent of those between 20 and 40 years, and 42.5 per cent of those over 40 years of age possessed a high titer of protective antibodies. The occurrence of antibodies at all ages agrees with the customary experience that in outbreaks of influenza individuals of all ages are attacked. The high incidence of antibodies in children and young adults constitutes further evidence that the human influenza virus is prevalent at the present time.

The interpretation of the results of protection tests with sera which afford partial protection to mice is at the present time impaired by lack of knowledge. Except when based upon history—and this is unreliable—observations regarding the persistence of anti-influenzal antibodies for more than 1 year have not been made. Furthermore, the presence of protective antibodies in the blood of adults does not indicate whether they were acquired as the result of a recent infection or whether they have persisted for years following an attack of influenza. That the partially protective action of a serum does not belong in the category of natural antibodies is shown by the fact that this capacity is not destroyed by heating the serum at 60°C. However, 2 cases of influenza have been observed in which the serum of the individual taken at the height of the disease exerted partial protection against the virus, but after recovery the serum completely neutralized the virus. These observations suggest that other individuals whose sera do not protect completely against the virus of human influenza may be susceptible to infection with the virus. Moreover, of 31 sera obtained from individuals who had suffered from influenza in 1934–35, 27, or 87 per cent, possessed sufficient antibody to neutralize the virus completely.

poliomyelitis virus deserve to be considered in this general relation. It was found that in neutralization tests performed with the serum of normal children or of children convalescent from poliomyelitis against the older passage strain of poliomyelitis, no difference could be detected between the effects of normal and convalescent serum. The results appeared to bear a closer relationship to age than to actual illness. When the same sera were tested against a more recently isolated strain, it was found that convalescent children's serum protected against this strain, while normal serum did not. The age differences in serum protection against the older strain of poliomyelitis virus are very similar to those noted by Shope (9) with the serum of human individuals tested against swine influenza virus. The high incidence of protective antibodies in the serum of recent poliomyelitis convalescents against the recently recovered strain of poliomyelitis is also similar to that obtained with the serum of recent influenza convalescents against the human influenza virus.

No attempt at a final interpretation of the epidemiological significance of the results of the present study can be made. Sufficient evidence regarding the persistence of immunity to human influenza has not yet been accumulated to justify detailed inferences concerning the prevalence of influenza in the past. The proportion of positive sera in young children certainly indicates a prevalence of infection due to the human influenza virus at the present time. This indication is strengthened by the recovery of strains of the virus during recent epidemics of human influenza, as well as by the fact that the serum of recently recovered individuals, in the great majority of instances, possesses a high titer of antibodies against strains of human influenza virus.

SUMMARY

The results of mouse protection tests with 136 human sera and a strain of human influenza virus are described. After the 1st year of life, the sera of approximately half the individuals tested contained sufficient antibody to furnish complete protection to mice. A much higher percentage of the sera obtained from individuals recently convalescent from influenza exerted a completely protective effect. On the other hand, certain sera protected only partially under the conditions of the tests.

action is observed with sera of children under 10 years of age, whereas practically all adult sera protect completely against the swine virus. The period of highest incidence of protective antibodies against swine influenza virus is the same (from 20 to 40 years) as that in which the combined percentage of complete and partial protection against human influenza virus is greatest.

The serum of 40 of 79 individuals over the age of 12 years exerted complete protection against both human and swine strains of influenza virus. On the other hand, certain sera protected completely against human (P. R. 8) virus, but not against the swine virus, and *vice versa*. It is seen, therefore, that in addition to the differences in the protective action noted in the serum of children when tested against the human and swine influenza viruses, specific differences are to be observed in the protective action of the serum of adult individuals tested against the human and swine viruses.

Studies (10) with the sera of animals convalescent from experimental infection with human or swine influenza virus, or animals hyperimmunized by repeated inoculations of virus, have shown that the immune response to the primary infection is specific for the virus employed. After repeated reinfections, however, the serum may develop a broader zone of activity, and may confer some degree of passive immunity to mice against the heterologous virus as well. This effect is most noticeable in animals repeatedly infected with the human influenza virus, and it suggests that the swine influenza virus possesses antigenic components which are also present in the human influenza virus as secondary antigens. It seems possible that a similar set of conditions may obtain in the human population. The serum of children 1 to 5 years old exerts a specific effect against the human influenza virus, owing presumably to a single attack. The serum of older children and adults who may have had repeated exposures to the human influenza virus might, on the other hand, have developed the broader immune zone which would then afford a certain degree of cross-protection against the virus of swine influenza. Conversely, if human individuals had been attacked by the virus of swine influenza, partial protection might be exhibited by the serum against human influenza virus.

The recent studies by Paul and Trask (11), with different strains of

THE INCIDENCE OF NEUTRALIZING ANTIBODIES FOR SWINE INFLUENZA VIRUS IN THE SERA OF HUMAN BEINGS OF DIFFERENT AGES

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The experiments described in an accompanying paper (1) were conducted in an effort to determine the factors involved in the development of heterologous neutralizing antibodies by various animals, following immunization or infection with the viruses of swine and human influenza. It was found that, while both human and swine influenza viruses were neutralized consistently by their homologous immune sera, the sera from animals convalescent from a single infection with one virus possessed little, if any, neutralizing capacity for the other. Repeated exposures of the animals to either virus, but especially that of human influenza, tended to increase the heterologous neutralizing activity of their sera.

In a second paper (2) the neutralizing action of sera from a group of human subjects of various ages on human influenza virus was reported. The present paper deals with the ability of these same sera to neutralize swine influenza virus, and the results will be compared with those of the preceding paper in an attempt to determine the relation of the swine virus to disease in man.

The strain 15 swine influenza virus was used in all of the present experiments. It was obtained originally through the kindness of Dr. Fred Crow from a case of the swine disease occurring in Iowa in December, 1930. The sources of the human sera employed have been given in the preceding paper. 11 of the 137 sera obtained were tested against human but not swine virus. 2 more sera, found satisfactory for use by the technique of inoculation employed by Francis and Magill (2) proved toxic for mice by the method used in this laboratory. 1 serum was tested against swine but not human influenza virus. The remaining 123 sera were tested for their ability to neutralize both human and swine virus and these

The results have been compared with those obtained by Shope in tests done with the same sera against swine influenza virus. The possible epidemiological significance of the results is discussed.

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TABLE I

Neutralization Tests with Swine Influenza Virus and Sera of Human Beings of Various Ages

Serum No.	Age of donor	Serum-virus mixture administered intranasally to mice — Pulmonary lesions — Mouse No.				Result
		1	2	3	4	
	days					
1	3	0*	0	0	0	P†
2	4	0	0	2+	0	P
3	5	0	0	0	0	P
4	6	0	2+	0	2+	I
5	6	0	0	0	0	P
6	7	0	0	0	0	P
7	7	0	0	0	0	P
8	8	0	0	0	0	P
9	10	2+	0	0	0	P
10	11	0	0	0	0	P
11	14	0	0	0	0	P
	mos.					
12	1	0	0	0		P
13	1		Toxic			
14	2	3+	3+	2+		NP
15	2					
16	3	4+‡	4+‡	4+‡		NP
17	8	4+‡	4+‡	4+‡	4+‡	NP
18	9	4+	4+	4+		NP
19	9	4+	4+	4+		NP
20	13	2+	3+	2+	3+	NP
21	13½	2+	3+	2+	2+	PP

* 0 = mouse with no detectable influenzal lesions postmortem.

1+ = mouse with influenzal pneumonia involving upwards to ¼ of lung at postmortem.

2+ = mouse with influenzal pneumonia involving from ¼ to ½ of lung at postmortem.

3+ = mouse with influenzal pneumonia involving from ½ to ¾ of lung at postmortem.

4+ = mouse with influenzal pneumonia involving from ¾ to all of lung at postmortem.

† P = complete protection—serum neutralized the virus.

I = incomplete protection—serum exerted considerable neutralizing effect on virus but failed to protect completely.

PP = partial protection—serum exerted slight neutralizing effect on the virus.

NP = no protection—serum failed completely to neutralize the virus.

‡ = mouse died.

form the basis for the present paper. It is realized that the number of sera studied is small statistically and that gaps exist in certain important age groups.

Neutralization Tests

The neutralization tests were performed as previously described for swine influenza virus (1). The supernatant of a 2 per cent suspension of glycerolated infected mouse lung was used as the source of virus and mixed with an equal amount of each serum to be tested. The period of storage of the mixtures, the method of their administration to mice, and the criteria for judging the neutralizing effect of sera of unknown potency were the same as already described (1). The amount of swine virus administered to each mouse in each serum-virus mixture was sufficient to kill all or most of the control mice within the 6 day period that each test was allowed to run. At the end of 6 days all surviving mice were killed with chloroform, and the extent of their pulmonary lesions recorded. These lesions and those of mice which died earlier were graded from 4+ for lungs exhibiting a complete influenza virus pneumonia, to 0 for those whose lungs were free of influenza lesions. The basis upon which the final result of each test was determined and upon which the degree of protection afforded by each serum was graded has been described in the preceding paper (2). Mice 3 to 5 weeks old and weighing from 10 to 15 g were used.

The results of experiments in which human sera were tested for their ability to neutralize swine influenza virus are shown in Table I.

Consideration of the data given in Table I and presented graphically in Text-fig. 1 shows that the sera of infants between the ages of 3 days and 1 month consistently neutralized swine virus. These results are in agreement with those with sera from individuals of the age group of the mothers of the infants and may be explained as probably due to maternal transfer of neutralizing antibodies. The sera of babies from 2 to 9 months of age, on the other hand, failed to neutralize swine influenza virus. 1 of the 14 sera from children between the ages of 1 and 5 years and 2 of the 8 from children 6 to 9 years old neutralized the virus of swine influenza completely or almost completely. Others of these age groups showed evidence of possessing small amounts of neutralizing antibodies and these will be discussed in more detail later. Of 7 sera from children between the ages of 10 and 12 years, 4 neutralized swine influenza virus completely or almost completely. Those from persons in the higher age groups, from 21 years on, with very few exceptions, neutralized the virus of swine influenza. The results of these experiments are in striking agreement with those published recently by Andrewes, Laidlaw, and Smith (3).

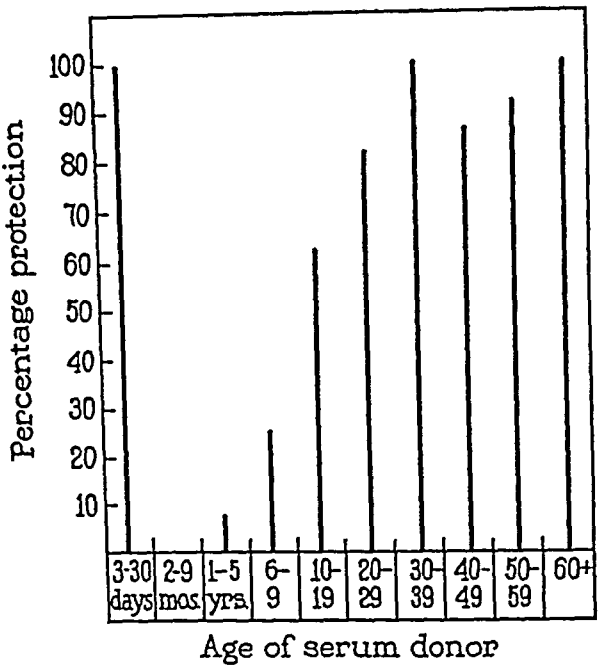
The curve in Text-fig. 1 representing the ability of sera from human beings to neutralize swine influenza virus rose steadily with advancing age to reach a peak of 100 per cent for the sera from persons in the 30 to 39 year age group. It de-

TABLE I—*Continued*

Serum No.	Age of donor	Serum-virus mixture administered intranasally to mice				Result
		Pulmonary lesions				
		Mouse No.				
		1	2	3	4	
	yrs.					
61	24	±	0	0	1+	I
62	24	0	1+	0	0	P
63	25	0	0	1+	0	P
64	25	0	2+	0	0	P
65	25	0	0	0		P
66	26	1+	1+	0	2+	PP
67	26	±	0	±	1+	PP
68	27	0	0	0	1+	P
69	27	0	1+	0	0	P
70	27	0	0	0	0	P
71	27					
72	28	0	0	0	0	P
73	28	0	1+	±	1+	I
74	28	0	1+	0		I
75	30	1+	0	0	2+	I
76	30	0	0	0	0	P
77	30	0	0	0	0	P
78	30	0	0	0	0	P
79	31	0	0	1+	0	P
80	31	0	0	0	1+	P
81	31	0	0	0	0	P
82	31	0	0	0	0	P
83	31	0	2+	1+		I
84	32	0	±	1+	1+	I
85	32	0	0	0		P
86	32	0	0	0	0	P
87	33	0	0	0		P
88	33	0	0	0		P
89	34	0	0	0	0	P
90	34	0	0	0		P
91	34	0	0	0	0	P
92	34	0	1+	1+	0	I
93	34	0	0	0	1+	P
94	35	0	0	0	0	P
95	36	0	0	0	0	P
96	36	0	0	0		P
97	37	0	0	0	0	P
98	40					
99	42	0	0	0	0	P

TABLE I—*Continued*

Serum No.	Age of donor	Serum-virus mixture administered intranasally to mice: Pulmonary lesions: Mouse No. 1	2	3	4	Result
22	*yrs.*	4+‡	4+‡	4+‡	4+‡	NP
23	1½	4+‡	4+‡	4+‡	4+‡	NP
24	2½	4+‡	4+‡	4+‡	4+‡	NP
25	3	3+	3+	3+	2+	NP
26	3	4+‡	4+‡	4+‡	3+	NP
27	3	2+	0	2+	0	NP
28	3	2+	2+	3+	2+	I
29	3	2+	3+	2+	2+	PP
30	3					PP
31	3	4+‡	4+‡	2+	4+	NP
32	3	2+	3+	2+	2+	PP
33	4	4+‡	4+‡	4+‡	4+‡	NP
34	5	2+	1+	2+	1+	PP
35	6	1+	0	0	0	NP
36	6	4+‡	3+	3+	1+	PP
37	6½	3+	2+	2+	1+	P
38	7	2+	1+	0	1+	NP
39	7	2+	1+	1+	1+	PP
40	8	2+	2+	1+	2+	PP
41	9	4+‡	4+‡	2+	4+	PP
42	9			4+		PP
43	9	0	0	0	P	NP
44	9					
45	10	0	0	1+	I	
46	10			4+‡	NP	
47	10	0	0	3+	PP	
48	11	4+‡	4+‡	±	NP	
49	11	2+	2+	0	I	
50	12	3+	3+	±	P	
51	12	±	0	1+	I	
52	12	0	±	0	P	
53	15	±	1+	2+	NP	
54	18	0	0	3+	P	
55	18		2+	1+	NP	
56	19	0	3+	0	P	
57	21	0	0	0	I	
58	22	0	2+	0	P	
59	22	4+‡	0			
60	23	0				



TEXT-FIG. 1. Percentage of persons of various ages whose sera neutralize the virus of swine influenza. For the purposes of this chart sera which give incomplete (see Table I) as well as complete protection are included.

Protection	Severity of pulmonary lesions (mice)	Age of serum donor									
		3-30 days	2-9 mos.	1-5 yrs.	6-9	10-19	20-29	30-39	40-49	50-59	60+
None	+++		•• ••	••• •••	•	•					
	++		•	• •	•	•	•				
Partial	++			•• ••	•••	•				•	
	+			•	•		• •		• •		
Incomplete	±	•		•		•••	• • • •	• • • •	• • • •	•	
Complete	0	••••• ••••• ••			• •	• •	•••••• ••••••	•••••• •••••• ••••••	•••••• ••••••	•••••• ••••••	•••••• ••••••

TEXT-FIG. 2. Degree of neutralizing activity of sera from persons of various ages for swine influenza virus in mice. Each dot represents a virus neutralization test with serum from one person.

TABLE I—*Concluded*

Serum No.	Age of donor	Serum-virus mixture administered intranasally to mice: Pulmonary lesions: Mouse No. 1	2	3	4	Result
100	*yrs.* 42	0	0	0	0	P
101	43	0	0	0	±	P
102	44	0	1+	0	1+	I
103	45	0	1+	2+	0	I
104	45	0	0	0	0	P
105	46	0	0	±	0	P
106	46	0	1+	0	0	P
107	46	0	0	0	1+	I
108	46	0	0	1+	1+	I
109	46	0	0	0	0	P
110	47	0	1+	0	0	
111	47	2+	0	0	0	
112	47	0	0	0	1+	
113	48	0	0	1+	1+	
114	49	0	0	0	0	
115	50	2+	2+	0	0	
116	50	0	1+	2+		
117	50	0	0	1+	±	
118	51	0	0	0	1+	
119	52	1+	0	±	0	
120	52	0	1+	0	0	
121	53	0	0	0	0	I
122	53	0	0	1+	0	P
123	54	0	1+	0	0	I
124	56	0	0	0	0	P
125	57	0	0	0	0	P
126	58	0	0	0	0	P
127	58		0	0		P
128	59	0		0	0	P
129	60	2+	0			P
130	60	0	1+	0		P
131	64	0	0	1+	1+	
132	65	0	0	0	2+	P
133	65	0	0	0		PP
134	66	0	0	2+	0	P
135	70	0	0	0	0	P
136	70+	0	0	0		P
137	73	0	2+	0	0	P
	76		0	0	0	P
		1+		0	0	P
			Toxic	0	0	P
		0	0		0	P
						P

Comparison of the Ability of Human Sera to Neutralize the Viruses of Human and Swine Influenza

There can be no doubt from the work of Smith, Andrewes, and Laidlaw (4) that the sera of persons convalescent from influenza neutralize their strains of the human virus. The value of the neutralization test as an indicator of the type of virus involved in previous human infections is suggested by its specificity in animal infections of known type (1, 4, 5, 6). However, it remained for Francis and Magill (7) to demonstrate conclusively that man actually develops antibodies neutralizing human virus following an attack of the disease. They found that the sera of 3 persons, bled during the acute stage of an attack of influenza, failed to neutralize the P. R. 8 strain of the virus of human influenza, whereas that obtained during their convalescence and again 6 months later did neutralize the virus.

The presence of antibodies in human sera capable of neutralizing swine influenza virus is more difficult to interpret because no strain of influenza virus, immunologically identical with that obtained from swine, has been recovered from man. A possible explanation for the presence of these antibodies in such a large proportion of the adult sera examined is afforded by the experiments recorded in the first paper of this series (1). It was shown that while serum of animals convalescent from a single infection with the virus of human influenza possessed little, if any, ability to neutralize swine virus, serum from animals submitted to repeated exposures to human virus was capable of partially or completely neutralizing swine virus. These findings suggested the possibility that the neutralizing properties of human sera for swine virus might be the result of repeated exposures to the virus of human influenza. The fact that sera from adults neutralized swine virus much more frequently than that from children was in accord with this possibility; conceivably the more advanced the age of the person the more numerous had been his opportunities for exposure to the virus of the human disease. The results of the neutralization test with swine virus alone are not sufficient to exclude this possibility. However, when the results of duplicate neutralization tests against the viruses of both human and swine influenza were compared, it was evident that, in a number of instances at least, neutralization of swine virus could not be considered the result of repeated exposures to the

clined slightly in age groups of the next two decades but this is of doubtful significance, since the 3 sera responsible for the decline all partially neutralized the virus.

Text-fig. 2 shows the neutralizing activity for swine influenza virus of sera from persons in the various age groups. The results recorded below the double line have been included in Text-fig. 1 and need no further discussion. Those above the double line represent results with human sera that either failed to neutralize swine virus or neutralized it only partially. The chart shows that most of the non-neutralizing sera were from persons less than 20 years of age. The serum of only 1 person above 20 years of age failed completely to neutralize the virus, while 5 others are recorded as partially neutralizing it.

Correlation of Past History of Influenza with Presence of Swine Influenza Virus-Neutralizing Antibodies

74 persons over the age of 12 years recorded in Table I were questioned as to their past influenza history. The sera of 44 out of 45 (97.7 per cent) of those giving a positive history of influenza neutralized the virus of swine influenza. 17 of those possessing a neutralizing serum gave as the date of their illness a time between 1918 and 1923, 12 between 1930 and 1935, and 5 had influenza both in 1918 and early in 1930. 10 were certain of having had an attack of influenza but were indefinite concerning the date; the 1 person giving a history of influenza whose serum failed to neutralize the virus of swine influenza fell in this group. 29 persons stated that to the best of their knowledge they had never had influenza. The sera of 25 of these (86.2 per cent), however, neutralized swine virus. Since it is realized that histories of influenza outside of pandemic periods are not accurate, the figures outlined above are believed to be of little significance.

Correlation of Age of Serum Donor with the Presence of Neutralizing Antibodies for Swine Influenza Virus

Of the individuals recorded in Table I, excepting the infants 1 month of age or younger, the sera of only 4 of 31 of those under 12 years of age neutralized the swine virus, whereas only 6 of 81 of those 12 years of age or older failed to do so. The possible significance of the correlation between age and the possession of neutralizing antibodies for swine influenza virus will be considered later. It is of interest that Andrewes, Laidlaw, and Smith (3), in their neutralization tests with English sera and swine virus found that none of their sera from persons under 10 years of age neutralized the virus.

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TABLE II

A Comparison of the Ability of Sera from Human Beings to Neutralize the Viruses of Human and Swine Influenza

Designation group	Degree of protection conferred by sera against each influenza virus		Age of serum donors										Comment and interpretation
	Human virus	Swine virus	3–30 days	2–9 mos.	1–9 yrs.	10–19 yrs.	20–29 yrs.	30–39 yrs.	40–49 yrs.	50–59 yrs.	60–69 yrs.	70+ yrs.	Totals	Type of virus involved in past influenza infections as judged by results of virus neutralization tests
			No.	No.	No.	No.	No.	No.	No.	No.	No.	No.		
1	NP*	NP	0	4	5	0	0	0	0	0	0	0	9	No past influenza infections
2	P	NP	0	1	4	1	0	0	0	0	0	0	6	Past influenza infections with human influenza (PR 8 type) virus
3	P	PP	0	0	5	0	0	0	0	0	0	0	5	
4	NP	P	1	0	0	0	1	2	2	3	1	1	11	Past influenza infections with swine influenza (strain 15 type) virus
5	PP	P	4	0	1	1	2	6	4	3	3	0	24	
6	P	P	5	0	1	1	7	10	2	4	1	2	33	Past influenza infections with both human and swine influenza viruses—or cross-immunity†
7	P	I	1	0	1	2	1	1	2	1	0	0	9	Past influenza infections with human influenza virus and waning immunity to swine influenza virus—or cross-immunity
8	I	P	0	0	0	0	0	1	1	1	1	0	4	Past influenza infections with swine influenza virus and waning immunity to human influenza virus—or cross immunity
9	I or PP	I or PP	1	0	2	1	3	3	3	0	0	0	13	Past influenza infections with either or both swine and human influenza viruses with waning immunity—or previous infection with one related but not identical to either type of virus
10	NP	I or PP	0	0	2	1	1	0	1	0	0	0	6	
11	I or PP	NP	0	0	1	1	1	0	0	0	0	0	3	
Totals.....			12	5	22	8	16	23	15	13	6	3	123	

*NP = no protection.
I = incomplete protection.
PP = partial protection.
P = complete protection.

These symbols are fully explained under Table I.

† Repeated exposures of animals to either virus, but especially that of human influenza, increase the heterologous neutralizing activity of their sera (1).

human virus. The sera from 35 persons in the group studied neutralized the swine virus completely but failed to neutralize that of human influenza (see Table II). If, in these 35 cases, the ability to neutralize swine virus had been the result of repeated infections with the virus of human influenza, it would be anticipated that the latter virus would have been neutralized also by the sera. The facts lead one to ask whether the human donors of sera which neutralized swine virus only had not undergone a previous infection with a virus of this sort. The results obtained in the present study of swine virus have been compared with those described by Francis and Magill (2) for human virus, in order better to evaluate their significance. This comparison is outlined in Table II. An interpretation of the findings, on the basis of the cross-neutralization experiments with sera from animals known to be immune to swine or human influenza virus (1), is also included.

As will be seen in Table II, the sera from only 9 persons, all under 7 years of age (group 1), failed entirely to neutralize either human or swine influenza virus. The sera from 6 persons (group 2), all under 12 years of age, neutralized the virus of human influenza but not that of swine influenza. The sera of another group of 5 persons (group 3), all under 8 years of age, neutralized human virus completely and also exerted a slight neutralizing effect upon swine virus. The sera from 11 persons (group 4), all, with the exception of one new-born, over 24 years of age, neutralized the virus of swine influenza but not that of human influenza. The sera from another group of 24 persons (group 5) neutralized swine virus completely and also exerted a slight neutralizing effect on human virus. 18 of the members of this group were over 24 years of age, 4 were new-born, and the remaining 2 were 6 and 12 years of age.

The sera of 33 persons, listed in Table II as group 6, completely neutralized the viruses of both human and swine influenza. With the exception of 5 new-born and 1 child 9 years old, all of this group were 18 years of age or older. The general age distribution was thus the same as for those listed in groups 4 and 5 whose sera had neutralized only swine influenza virus. The sera of 9 persons, designated as group 7 in Table II, neutralized human influenza virus completely and also exerted considerable neutralizing effect on swine virus, while the sera of 4 others, designated as group 8, neutralized swine virus com-

individuals studied neutralized swine virus but not human virus. It seems unlikely that the age distribution of antibodies neutralizing swine influenza virus can be interpreted on the basis of frequency of opportunity for infection with a virus that is at present widely prevalent. Furthermore, the age distribution of antibodies found by Francis and Magill (2) for an influenza virus of human type known to be prevalent in man during the past 2 years is quite different from that for the virus of swine influenza.

The history of swine influenza furnishes a clue to the interpretation of the neutralization experiments under discussion. The disease was first recognized as a clinical entity in the late summer or fall of 1918.[1] Conversations with veterinary practitioners in eastern Iowa have revealed that the disease caused serious losses among swine on exhibition at the Cedar Rapids Swine Show held from September 30 to October 5, 1918. At the conclusion of the show, the swine, many of them ill, were returned to their home farms and, within 2 or 3 days of their return, influenza was stated to be rampant in the portion of the drove that had remained at home. Shortly thereafter the disease became widespread among swine herds in Iowa and other parts of the Middle West. It persisted in various localities until January of 1919. The epizootic in the autumn and winter of 1919 was stated to be as extensive and severe as that in 1918. The disease has appeared among swine in the Middle West every year since but varies from year to year in its severity and extent.

According to Dorset, McBryde, and Niles (11), Dr. J. S. Koen, an Inspector in the Division of Hog Cholera Control of the Bureau of Animal Industry, was the first to recognize the disease as being different from any previously encountered. He was so much impressed by the coincidental prevalence of human influenza and by the resemblance of the symptoms seen in man to those occurring at the time in hogs that he became convinced that the two were actually the same. He therefore gave the name of "flu" to this new disease of hogs. The opinion of Koen that "flu" represented an entirely new swine

[1] Dr. Grant B. Munger of Cedar Rapids, Iowa, has stated in a personal communication that he observed herds of swine ill with influenza as early as August of 1918 in western Illinois where he was then serving as an inspector in the Division of Hog Cholera Control of the Bureau of Animal Industry.

pletely and exerted considerable neutralizing effect on human virus. The sera from persons in the last three groups in Table II neutralized neither virus completely, but did partially protect against one or both of them.

The comments on the possible significance of these data in indicating the type of virus involved in past influenzal infections of the persons studied, made in the last column of Table II, are self-explanatory. It is clear that antibodies neutralizing swine influenza virus are present in human sera and frequently independent of those effective against the human virus. The most evident explanation of their presence is that they arose as a result of previous infection by a virus whose antigenic composition was similar to that of swine influenza. The high incidence of swine virus-neutralizing antibodies in sera from adults and their rarity in sera from children further suggest that the agent responsible for their generation has not recently been widely prevalent. This will be more fully discussed later.

DISCUSSION

So far as the present studies are concerned, it has been found that the sera from a very high proportion of human adults neutralize swine influenza virus while those from children below the age of 12, with the exception of new-born infants, seldom exert such an effect. On the surface, the situation would appear to be similar to that known for diphtheria, for instance, in which the serum antitoxin titer, low in childhood, increases with advancing age. To be entirely comparable, however, the causative agent, namely an influenza virus of an antigenic composition similar to swine virus, should be rather widespread throughout the human population. The viruses isolated from clinical cases of influenza in man during the past 2 years from such widely separated localities as London (*5*), Puerto Rico (*9*), Philadelphia (*6*), and Melbourne (*10*), are immunologically identical (*3, 6, 10*). Since the human virus differs immunologically from the swine virus (*1, 3, 4, 6*), its presence cannot be held accountable for the high incidence of antibodies for swine influenza virus encountered in sera from human adults. Moreover, as has been pointed out earlier, the presence of such antibodies cannot be considered the result of repeated exposures to the current human type of virus, because the sera from 35 of the

further suggests that its past activity, so far as man may be concerned, lay in the production of influenza.

All of these facts viewed as a whole make it necessary to consider seriously the theory that swine influenza virus represents a surviving form of the human pandemic virus of 1918, and that it has not had its immunological identity detectably altered by its prolonged sojourn in hogs. On the basis of this assumption the presence in human sera of antibodies neutralizing the swine virus would be considered as indicating that the donors of these sera had undergone an immunizing exposure to or infection with an influenza virus of the 1918 pandemic type.

Andrewes, Laidlaw, and Smith (3) in interpreting the significance of their neutralization experiments with human sera obtained in England, and the same strain of swine virus used in the present experiments (strain 15, Iowa, 1930) have guardedly suggested an explanation similar to that just outlined. They have qualified their interpretation by considering the possibility that the antibodies to swine virus in adult human sera may be non-specific in the sense that they represent past contact, not with that virus, but with some unknown related antigen.

If swine influenza virus is actually a surviving form of the 1918 human pandemic strain, then two inferences, interesting from an epidemiological standpoint, become apparent immediately. The first of these is that virus of the 1918 type has been present in human beings within the past 6 years, since the serum from one 6 year old child in the group tested neutralized the swine virus. The second is that persons at present susceptible to virus of the 1918 type, as indicated by the failure of their sera to neutralize swine influenza virus, are limited largely to those in the lower age groups born since pandemic influenza ceased to be prevalent.

SUMMARY

Sera from a very high proportion of the human adults and new-born infants studied neutralized swine influenza virus; sera from children below the age of 12 years seldom exerted such an effect. The results of neutralization experiments with human sera and the virus of swine influenza have been compared with the outcome of similar tests with

epizootic disease, not seen before 1918, was shared by many veterinary practitioners in the Middle West. Dimoch, in an exhaustive paper on the differential diagnosis of diseases of swine (12) presented in August, 1918, makes no mention of a disease of swine bearing any resemblance to influenza. It seems clear that swine influenza appeared in the Middle West as an epizootic disease for the first time, in recent years at least, during the late summer or early autumn of 1918.

The new disease thus made its first appearance at a time when human pandemic influenza was at its height in the Middle West. Many thought that the two diseases were connected and that swine might have been infected in the first instance from human beings (13). Murray and Biester (14) have called attention to the similarity existing between the "water logged" lung of the human influenzal pneumonia of 1918 and that of the pneumonia of fatal swine influenza. The writer, in earlier work (15), was impressed not only by similarities between the clinical and pathological pictures of human and swine influenza but by the association of a leucopenia with both diseases and, most especially, by the similarity of the predominant bacterium encountered in each disease: *H. influenzae* in the epidemic disease of man and *H. influenzae suis* in the epizootic disease of swine. On the basis of the similarities between the two diseases, after establishment of the fact that swine influenza was caused by the combined action of a filtrable virus and *H. influenzae suis*, it was suggested that an investigation of the possibility that Pfeiffer's bacillus and a filtrable agent act in concert to cause influenza in man seemed indicated. The possibility received support from the discovery by Smith, Andrewes, and Laidlaw (5) of a virus in cases of human influenza similar to that etiologically important in swine influenza, and Laidlaw (16) propounded the view that "the virus of swine influenza is really the virus of the great pandemic of 1918, adapted to the pig and persisting in that species ever since."

The ability of such a large proportion of the sera from human adults to neutralize the virus of swine influenza adds weight to the view that this virus or one of its antigenic composition has recently been widely prevalent in man. The similarity of swine influenza virus to that etiologically important in recent influenza in man, with regard to its pathogenic activity in common experimental animals (5, 8, 9, 17, 18),

IMMUNOLOGICAL STUDIES WITH THE VIRUS OF INFECTIOUS LARYNGOTRACHEITIS OF FOWLS USING THE DEVELOPING EGG TECHNIQUE

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PLATE 46

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It has previously been shown (Burnet (1)) that the virus of infectious laryngotracheitis of fowls can be readily propagated on the chorioallantoic membrane of the developing egg, producing in this situation lesions characterised by ectodermal proliferation and the presence of intranuclear inclusions.

The recognition of the disease in New South Wales by Seddon and Hart (2) has stimulated further work on these lines with strains of Australian origin. The lesions produced have been studied in greater detail than previously in the light of further experience with the developing egg technique, and a method of detecting the corresponding antibodies has been developed which has proved of great value in studying the epidemiology of the disease.

Material and Methods

Several strains of virus have been used. Strains F and G were received through the courtesy of Dr. H. R. Seddon, Director of the Glenfield Veterinary Research Station, New South Wales. Strain F has been used in the great majority of the experiments; strains S1 and S2 are Victorian strains, obtained from two fowls showing typical symptoms and post mortem findings. Both came from a poultry farm in one of the outer suburbs of Melbourne. Although the symptoms were typical, this virus appears to possess only a low grade of virulence since only a few deaths which might be attributed to it have occurred on the farm. The New South Wales strains, on the other hand, were isolated from fairly severe epizootics. An American strain B.A.I. was also available which had been received by Dr.

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the virus of human influenza, and it seems evident that the presence of antibodies neutralizing swine influenza virus cannot be deemed the result of repeated exposures to the current human type of virus. From the known history of swine influenza and the similarity of its etiologic virus to that obtained from man it seems likely that the virus of swine influenza is the surviving prototype of the agent primarily responsible for the great human pandemic of 1918, as Laidlaw has already suggested. The presence in human sera of antibodies neutralizing swine influenza virus is believed to indicate a previous immunizing exposure to, or infection with, an influenza virus of the 1918 type.

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duce recognisably different focal lesions. The first lesions obtained were regarded as almost certainly due to fowl pox virus. They show the same uniform flat conical proliferation without the necrotic central ulcer of the first group. The size of the foci varies considerably from egg to egg. Though large forms occur there is a distinctly lower average diameter than is found with the New South Wales strains.

With undiluted material the confluent or semiconfluent lesions have the character which would be expected from fusion of the constituent foci. In addition there is often free fluid lying on the surface of the membrane and always a considerable degree of oedematous thickening.

If the eggs are left for 5 or 6 days after inoculation, death of the embryo is usual, probably mainly from interference with respiration by the large membrane lesion. No virus has been detected in the embryo organs in such instances.

The histology of the lesions will not be discussed in detail. With all strains there is a primary infection of ectodermal cells with associated proliferation and the appearance of intranuclear inclusions in almost every cell. The stage of proliferation is followed by degenerative vacuolation or massive necrosis of the infected cells and the appearance of inflammatory mesodermal changes. The New South Wales strains F and G show earlier and more extensive necrosis than the Victorian ones. The piled up lesion of the latter strains contains many vacuolated and degenerate cells, but there is not the acute necrosis and desquamation which occurs in the central ulcerated area of F, G and B.A.I. strain lesions.

Maintenance of Virulence and Infectivity of Strains during Passage on Egg Membranes

Through the cooperation of Dr. Seddon egg membrane lesions of several strains were tested for infectivity in susceptible cockerels.

The membranes were removed to glycerol-saline and packed on ice during transit to Glenfield Research Station, New South Wales. On the following day emulsions were prepared and intratracheal inoculations performed. Three New South Wales strains F (25th egg passage), G (3rd passage) and Sa (2nd egg passage), one Victorian S1 (14th egg passage) and the American strain B.A.I. (3rd passage) were tested. Strains F, G, Sa and B.A.I. all produced severe infections, with two deaths amongst the 20 birds used. The Victorian strain S1 gave less

Seddon from Dr. J. R. Mohler, Head of the Bureau of Animal Industry. Egg passages were initiated by inoculating the eggs with membrane filtrates made from the laryngeal and tracheal lesions. An Elford type of gradocol membrane of approximately 0.8 μ A.P.D. was used for filtration. The technique of egg inoculation has been several times previously described (Burnet and Galloway (3)). The only modification since introduced has been the substitution of eggs incubated for 12 days before inoculation in place of 10 day eggs. The older eggs are much less liable to show non-specific traumatic lesions and give more readily counted focal lesions. Once a satisfactory lesion has been obtained, there is no difficulty in maintaining continuous passage from egg to egg. The membranes retain their activity well when stored in 50 per cent glycerol-Ringer solution in the refrigerator.

Chorioallantoic Membrane Lesions Produced by Different Strains of Virus

The usual method of preparing an inoculum has been to grind up a freshly removed membrane showing typical lesions with sterile quartz powder and about 1 cc. of distilled water. The emulsion is then diluted with 5 cc. of ordinary nutrient broth and centrifuged to remove quartz, and tissue fragments. When the supernatant fluid is inoculated on to an egg membrane in 0.05 cc. amount, a confluent lesion results covering most of the available surface. If the fluid is diluted 1:10 or 1:100 in saline discrete foci appear. A 1:100 dilution will almost invariably provide a reasonably countable number of foci (from 10 to 100). The number of foci is at least roughly proportional to the concentration of virus added.

These foci can be regarded as analogous to bacterial colonies or more closely to bacteriophage plaques, and like them can be used both for qualitative characterisation of the virus and for its quantitative estimation.

The appearance of the foci divides the strains of infectious laryngotracheitis with which we have worked into two groups. The first comprises all the epizootic New South Wales strains and the American strain B.A.I. The foci after 3 days incubation are usually 2 to 4 mm. in diameter and of characteristically irregular outline. Adjacent foci readily fuse into confluent areas. Two zones can be clearly seen in each focus, a central necrotic area darker and slightly yellowish in colour, and a peripheral zone of active ectodermal proliferation whiter and more opaque. Around the focus there is a grey haze of cellular reaction and a variable degree of oedema. The Victorian strains pro-

The use of measured quantities entails a slight modification of the egg inoculation technique described by Burnet and Galloway (3). Quantities of 0.05 cc. are dropped vertically on to the chorioallantoic membrane from a capillary pipette previously calibrated to 0.05 cc. with a weighed drop of mercury. Between each inoculation the pipette is sterilized by washing out in boiling distilled water and drying in a Bunsen flame. In order to avoid loss of fluid on to the shell membrane, the opening in this is made more extensive than usual and a flap is held back by a needle point, while the inoculum is blown out on to the chorioallantoic membrane. Fully developed 12 day embryos only should be used.

Eggs are opened after 3 days further incubation. The inoculated area is removed with scissors, the cut being made about 5 mm. outside the margin of the artificial air space. The membrane is then examined in saline against a dark background and the foci counted. If there is any difficulty in making an accurate count the membrane is stretched out on black paper wet with formalin-saline for a few minutes and then returned to a Petri dish of saline. The membrane lesions can be more readily examined and counted after this procedure.

If the individual foci are fairly small, up to 100 can be counted on a membrane, but with large foci any count over 40 or 50 will include a number of fused or semifused areas. Sometimes a fairly accurate estimate of the number of component foci in these areas can be made, but on other membranes a fused area may show no indications of the individual foci and no estimate is possible. In the tables an unqualified number indicates that the foci were readily counted and that any uncertainty would not make a greater difference than 5 per cent of the total count. $\pm$ after a number signifies that although fused areas are present a combined count and estimate is possible which is probably within 20 per cent of the true value. A number followed by + is used to describe a fairly common type of unsatisfactory membrane lesion in which the inoculum fails to spread satisfactorily. There is a central infected patch usually associated with a non-specific ulcerated lesion, and a number of adjacent more or less discrete foci. The number of foci which can be clearly distinguished is stated, but it is usually quite impossible to make any reasonable guess at the number of foci represented by the central area. If care is taken to use eggs which as judged by transillumination have reached the full normal development corresponding to 12 days' incubation, fewer of these unsatisfactory membranes are obtained. Confluent lesions are indicated +++, semiconfluent ++, and eggs dead usually from bacterial contamination, without specific lesions, by the usual sign †. There must always be a number of uncontrollable factors in these titrations and a fair proportion of eggs give quite unusable results. Fortunately, it is usually possible to tell from the character of the lesion whether the apparent count is likely to give a true measure of the virus content of the inoculum. As an example of the limitations of the method a fairly detailed account may be given of the results obtained when ten eggs were inoculated with 0.05 cc. amounts of a normal serum-dilute virus mixture. The eggs were inoculated in three batches immediately after mixing and at 1 and 2½ hours later. No change in titre apparently occurred over this

regular results but all the fowls inoculated were subsequently immune to inoculation with virulent tracheal exudate of strain F infections.

Birds experimentally inoculated with strains F, G, and Sa and B.A.I. were placed in contact with a normal fowl and in each case the contact bird developed symptoms, usually mild, and was subsequently shown to be immune to intratracheal inoculation with active virus.

TABLE I

Results of Inoculating Egg Membrane Lesions Intratrachcally in Susceptible Fowls

Virus F		
Saline emulsion 1:3	Symptoms 4th day	
Glycerol-saline emulsion 1:3	" 3rd "	Peracute type*
1:30	"	" " Death
1:300	" 4th "	" " 4th day
1:3000	" 6th "	" "
Nil		" "
Virus S1		
Saline emulsion 1:3	Nil	
Glycerol-saline emulsion 1:3	Symptoms 6th day	Immune 14 days later
1:30	" 6th "	"
1:300	Nil	" 14 " "
1:300	? Slight symptoms 6th day	" 14 " "
		" 14 " "
		" 14 " "

* For description of clinical types of infectious laryngotracheitis as seen in the current epizootics in New South Wales, see Seddon and Hart (2).

Details of the results of inoculation with material from eggs infected with strains F and S1 are shown in Table I.

These results indicate conclusively that the filterable agent propagated on the chorioallantoic membrane retains a full capacity to induce the typical disease in young birds even after 25 passages.

Technique of "Pock Counting" Titrations and Limitations of the Method

The immunological experiments to be described are based on the use of the number of macroscopically visible foci (pock count) as a measure of the amount of active virus present. In principle the method is closely analogous to the plating methods for titrating bacteriophages, but it is naturally more difficult to obtain consistent results.

position seems to be that if experiments are done in triplicate, 2 of the 3 eggs will probably show satisfactory counts and that the average count of the 2 will be within 50 per cent ± of the real count determined by the use of a large number of eggs. This is not a high degree of accuracy but it is a great deal more accurate than any current method of virus titration which does not use prohibitive numbers of animals.

The application of this technique to serum neutralisation tests is obvious. In practice the usual procedure has been to use a freshly prepared supernatant fluid from an infected egg membrane emulsion as stock virus. This is mixed usually diluted 1:10 with an equal volume of the immune serum, and after standing for an hour at room temperature 0.05 cc. quantities are inoculated into developing eggs. A dilution in saline (1:100) mixed with known normal hen serum

TABLE II

Pock Counts Obtained from 0.05 Cc. Inoculations of a Suspension of Virus B.A.I. in the Dilutions Shown

Dilution of virus	Pock counts
1:100	205, 184+, †
1:200	108, 93±, 46+
1:400	47±, 48, 19
1:1000	22, 29, †

provides one control, and a mixture of undiluted virus with a known immune serum another. All reagents are of course bacteriologically sterile.

Antigenic Identity of Laryngotracheitis Strains of Different Origin

No evidence has been obtained to suggest any qualitative antigenic differences amongst the strains we have worked with. From the appearance of the foci on egg membranes and from their known degree of virulence, the strains can be divided into two groups, the first comprising all the New South Wales strains and the American strain B.A.I., and the second the two Victorian strains. Antisera were available from birds immunised either naturally or experimentally with New South Wales virus and from three fowls which had been inoculated intratracheally with Victorian virus without showing symp-

period at room temperature. The following counts are arranged in the order in which the eggs were inoculated.

(1) 53+. Isolated foci with a small central fused lesion of perhaps 20 foci.

(2) 57+. Large foci with heavy semiconfluent accumulation at the center.

(3) A single fused central patch of small area and quite uncountable, probably due to failure of drop to spread.

(4) 64. Typical foci.

(5) 89. Well distributed large foci.

(6) 62±. Small foci with two semiconfluent patches near periphery.

(7) About 300 small foci fairly well distributed, except for two confluent patches. Foci are of type expected at about 40 hours after inoculation and presumably in large part represent secondary foci due to virus particles developed in the egg within the first 24 hours. This is the only egg inoculated with infectious laryngotracheitis in which this has been recognized, but the phenomenon is common on membranes inoculated with ectromelia or louping ill viruses.

(8) 60±. Large foci with one semiconfluent patch.

(9) 47+. Large moist patches at center, probably representing a fairly small number of foci.

(10) Embryo dead, contaminated.

Of the 10 eggs, 5 give countable membranes with 89, 64, 62, 60, 57 foci, 2 others are obviously of the same order 53+, 47+, while 3 for different reasons are completely unsatisfactory. In another series of 8 eggs inoculated with a uniform amount of virus the counts were 99, 95, 79, 65, 36: 25+, 25+, 16+. The last 3 eggs all showed large fused central lesions.

Within the limits of experimental error the number of foci appearing is directly proportional to the amount of virus added. Table II shows the result of a detailed titration of an emulsion of strain B.A.I., the dilutions being made in 10 per cent normal fowl serum in saline. For this experiment readings were made after 2 days incubation in order that the smaller foci might allow counts to be made from the more concentrated suspensions.

In this experiment the average count with 1:1000 dilution is approximately one-eighth that of the 1:100 dilution instead of one-tenth. The difference may well be fortuitous, but in one or two other instances a similar tendency to high values in the more highly diluted samples is evident and it may mean that the relation between plaque count and bacteriophage concentration which was described by Dreyer and Campbell-Renton (4) is also exemplified in the present technique.

These results are typical. Sufficient material for statistical treatment has not been accumulated but a reasonable statement of the

infecting susceptible cells and more readily removed by phagocytosis. It was generally recognised that no strictly virucidal effect of immune serum could be demonstrated. Sabin (5) has recently attacked this point of view, mainly on the basis of experiments in which reactivation of apparently inactive virus-serum mixtures was obtained by simple high speed centrifugation with resuspension of the sedimented virus particles. He holds that there is no evidence that antiserum has any effect on virus *in vitro*, its only action is to render the susceptible cells less ready to allow multiplication of the virus.

In reading Sabin's papers or any other work dealing with vaccinia-serum titration on the skin of the rabbit one is impressed with the unsatisfactory character of the titration method. The end-point is usually a small erythematous lesion not unlike many non-specific lesions and a small difference in individual judgment might readily result in the same experimental results being interpreted as showing no difference from the control or as showing a 90 per cent reduction in titre. When the titration method uses the death of an animal as the end-point, individual variability will also usually leave a possible error of one tenfold dilution when the usual two animals to each dilution are used. The use of a more accurate titration method therefore seems to be particularly needed in regard to this problem of the nature of the virus-antibody reaction.

The only thoroughly satisfactory quantitative work in this field is that of Andrewes and Elford (6) on the bacteriophage-antiphage relationship. While most bacteriologists still seem chary of accepting bacteriophages as truly autonomous viruses few will contest the statement that they behave in regard to susceptible bacteria exactly as if they were viruses, and one would expect that the effect of antisera would be very similar in the two cases. The use of laryngotracheitis virus and the egg membrane technique allows experiments to be made along precisely similar lines to those adopted in bacteriophage work and an attempt has been made to determine whether the salient features established by Andrewes and Elford for bacteriophages hold for laryngotracheitis virus and homologous antisera. Their chief conclusions were: (*a*) The inactivation process progresses with time, rapidly at first and then more slowly, but probably never reaching a permanent equilibrium value. (*b*) With a given concentration of

toms. These, initially free from antibody, developed very active antisera, one of which H5 was used in the experiments to be reported. The two others were qualitatively similar. It was found that serum H5 was a good deal more active than the New South Wales antiserum 326 which was used in most experiments, but, if the two sera were rendered comparable by using 326 undiluted and H5 diluted 1:5, no qualitative differences could be detected. There seems however, to be a real difference in the ease with which different strains can be neutralised by serum, the Victorian strains being more resistant than those of the more typical group. Table III shows the result of a comparison between strain F (New South Wales) and strain S2 (Victorian) the same sera being used and the conditions of experiment similar.

TABLE III

Cross Neutralisation Tests with New South Wales and Victorian Strains of Infectious Laryngotracheitis Virus

Virus strain	Final dilution	Serum and final dilution		
		326 Anti-F (1:2)	H5 Anti-S2 (1:10)	Normal fowl (1:10)
S2 (Victorian)	1:2	++, ++	++, ++	(40,000)
	1:20	41, 38, 37	55, 60±, 52±	(4000)
	1:800			106, 100±
F (N.S.W.)	1:2			(24,000)
	1:200	31, 20, 100±		108+, 137±
	1:2000		37, 55, 21	22, 26

In this experiment both virus strains were inactivated to a greater extent than was observed in earlier experiments of the same type but the relative resistance of the Victorian strains has always been clearly evident. The American strain and the other New South Wales strains resemble strain F.

The Process of Virus Inactivation by Immune Serum

The nature of the virus-immune serum interaction has been a matter of controversy for many years. On the whole there has been a general tendency to consider the reaction as resulting in a loose antigen-antibody union which rendered the virus particles less capable of

the next section the percentage law can be tested over only a relatively small range of virus concentrations, *viz.* those giving with immune serum a countable number of foci when the mixture is inoculated undiluted on to an egg membrane. Several experiments with different sera have all given results consistent with the view that the number of foci is within the limits of experimental error proportional to the concentration of virus. Table V summarises the most satisfactory of these experiments.

(*3*) *Reactivation by Dilution.*—If a mixture of virus and immune serum after standing 1 hour at room temperature is inoculated undiluted on to egg membranes and portion of the remainder immediately

TABLE V

Relation between Virus Concentration and the Number of Foci Produced from Immune Serum-Virus Mixtures

Serum final concentration	Time of contact	Final concentrations of stock virus emulsion in mixtures				
		1:2	1:4	1:8	1:16	1:200
	hrs.					
Immune 1:4	1–1¼	21±, 26, 8	11, 1+, †	5, 4, 2		
" 1:10	2¼–2½		27, 25, 19±	9, 12, 8+	7, 4±, 5	
Normal 1:4	3					40+, 62, 64, 59±, 16

Virus: B.A.I. (American) strain. Serum: H5 from a fowl immunised with strain S2.

diluted 1:10 in saline and the same standard volume of this also inoculated into eggs, both counts are of the same order of magnitude. The following protocol illustrates this reactivation by dilution.

Virus F undiluted plus serum 326 tested undiluted at 1 hour, 33, 36, 53 foci, the same mixture diluted 1:10 in saline and tested at once, 40, 9+, 18+ foci. Virus diluted 1:100 plus normal serum 31, 38, 34 foci. In another test with similar reagents the undiluted mixture at 1 hour gave 27, 8 foci a 1:10 dilution from this 22, 4+ foci.

(*4*) *Diminished Filterability of Virus in the Presence of Small Concentrations of Immune Serum.*—Two glycerolated egg membranes stored for a week in the refrigerator were ground with about 15 cc. of

serum the percentage of phage inactivated under uniform conditions is independent of the concentration of phage (the "percentage law"). (*c*) With some phages reactivation by dilution can be demonstrated. (*d*) Phages in the presence of small amounts of antiserum fail to pass membrane filters which are normally readily permeable to them. (*e*) Partially inactivated phage when brought into contact with susceptible bacteria only begins to multiply after a delay, for this reason producing abnormally small plaques on agar. All of these points have been studied with laryngotracheitis virus.

(*1*) *Time Course of Neutralisation by Serum.*—A large number of experiments have been made with various strains of virus and different serum concentrations and all have shown that the neutralisation effect is not immediately complete but runs a course very similar to that observed in bacteriophage work. Mixtures of virus with normal serum retain their original titre for at least 3 or 4 hours at room temperature (see section 3). The experiment, a protocol of which is given in Table IV, is typical.

TABLE IV

Influence of Time of Contact in Vitro on Results of Inoculating Serum-Virus Mixtures

Serum	Virus B. A. I.	Foci obtained when mixtures were tested at the times shown		
		Immediately	1 hr.	2 hrs.
Normal fowl	1:100	60, †		
	Undiluted	++, ++, (2–300)	80, †	76, 77, 12+, 20+
Immune 326	1:10	64, 51	8, 15	31

With serum 326 four other experiments gave results of the same order. With a more active serum H5 the results were less striking, the great bulk of the inactivation being apparent in the "immediate" titration. With an inoculum corresponding to about 3000 foci, serum-virus mixtures gave the following figures: immediately 9, 9; at 1 hour, 4, 5, 4; at 3 hours 1, 1.

(*2*) *The Percentage Law of Andrewes and Elford.*—It has been evident throughout that the number of foci produced with an immune serum was at least roughly proportional to the activity of the virus preparation used. Owing to the "dilution phenomenon" to be described in

branes have the appearance shown in Fig. 1. One set of membranes illustrates a titration with virus dilutions in normal serum and the other set the three membranes from the same experiment inoculated with virus partially inactivated by immune serum. The appearance of the lesions is entirely compatible with the view that there is a variable and often considerable (1–2 days) delay before the effective virus particles actually initiate the focal lesions.

DISCUSSION

The technique for propagating certain viruses on the chorioallantoic membrane of developing chicks by Goodpasture and his collaborators (7) is a method of great potential value in virus work. Its present application to the study of laryngotracheitis infections as they occur in Australia has indicated its usefulness in this sphere. Certain problems must be studied directly on the susceptible animal, but a great deal of virus investigation will always consist of estimations of the amount of virus in a given preparation or of the virus inactivating power of a given serum. For such work the only requisite is a suitable indicator organism susceptible to the action of the virus, and for many viruses, particularly those responsible for bird infections, the egg membrane technique offers many advantages. Laryngotracheitis virus is perhaps that for which its advantages are most easily demonstrable. In the first place it avoids the difficulties and expense of maintaining isolation between different experimental groups of birds and eliminates all complications due to unsuspected mild infections in supposedly normal birds. Within the limits discussed above all eggs are uniformly susceptible even to weak viruses which almost regularly produce only inapparent infections in experimental birds, and which probably could not otherwise be studied. The pock counting titration method allows quantitative experiments of much higher accuracy than could be attained with classical technique and the easy production of virus free from contaminating bacteria or other viruses might become of great value should vaccination of flocks become an economic necessity. Finally, our experience in Australia suggests that the use of the neutralisation test described is a necessary part of any survey of the extent of latent laryngotracheitis infections amongst poultry. Its application to such work will be described elsewhere.

saline and nutrient broth (about equal parts of each). The emulsion was centrifuged for 15 minutes in a small angle centrifuge and the supernatant passed through a sand and paper pulp filter. Two 5 cc. amounts of the filtrate were added respectively to 0.1 cc. of normal fowl serum and to 0.1 cc. of immune serum H5. The mixtures were shaken and then placed in the refrigerator for an hour and a half.

Two Elford type gradocol membranes of approximate A.P.D. 0.8 μ, both discs cut from the same sheet, were set up and about 4 cc. of each mixture filtered. Both showed some blocking of the filter, toward the end rather more with the immune serum than with the non-immune. As soon as filtration was complete, the membrane filtrates and the portions of the original sand and paper pulp filtrates which had been

TABLE VI

Influence of Dilute Immune Serum on Filterability of Laryngotracheitis Virus

	Virus and normal serum 1:50			Virus and immune serum 1:50		
	Undiluted	1:10	1:100	Undiluted	1:10	1:100
Before membrane filtration	+++, +++	(500), (500)	56, 33	+++, ++	170±, 26+	22
After membrane filtration	250±, 250±	28, 11	1, 2	0, 0, 0	0, 0	

retained, were chilled in iced water and tenfold dilutions made into cold saline containing a trace (Ringer proportions) of calcium chloride. Eggs were inoculated as soon as practicable thereafter. The membrane lesion counts after 3 days' incubation are given in Table VI.

Just as Andrewes and Elford found with bacteriophage-serum mixtures, an amount of immune serum having very little effect on the infectivity of the virus completely prevents its passage through a gradocol membrane filter normally permeable to it.

(5) *Diminution in Average Size of Focus with Partially Inactivated Virus.*—Membranes which have been inoculated with incompletely inactivated virus-serum mixtures characteristically show foci of smaller and less uniform sizes than are obtained with the control preparations lacking immune serum. Occasionally one finds well developed, nearly uniform foci in such circumstances but far more commonly the mem-

tiveness of a given degree of coating to prevent infection by a virus particle varies from one susceptible tissue to another, and if the degree of coating with antibody molecules is wholly or in part determined by a reversible reaction depending on the concentration of free antibody molecules, a reasonable interpretation of the phenomena is available which is quite harmonious with current views in other fields of immunology (Marrack (11)).

The resemblances between viruses pathogenic for animals and bacteriophages have been adequately described by d'Herelle and many others. The chorioallantoic membrane technique by offering an effective if rather inaccurate plating method allows a further striking series of resemblances between virus pocks and bacteriophage plaques to be noted. Even the detailed correspondence between phage and virus immunological reactions may only be indicative of superficial resemblances but the cumulative psychological effect of the correspondence is considerable. Studies of the fixation of viruses by susceptible cells carried out by Rous, McMaster and Hudack (12) have established another point of resemblance between the two agents, and, taken along with the present immunological studies of what is probably a typical virus, they suggest strongly that the attack on susceptible cell or bacterium must follow very similar lines in each instance.

SUMMARY

(1) The technique of chorioallantoic membrane inoculation has been applied to the study of the virus of infectious laryngotracheitis as it occurs amongst Australian poultry.

(2) When suitably diluted suspensions of virus are inoculated, isolated foci or pocks appear whose macroscopic form and histological structure is characteristic. The numbers of these foci may be used as a measure of the amount of virus present.

(3) Two distinct types of focus are produced by laryngotracheitis strains, one being characteristic of epizootic strains from New South Wales and from America, the other of a Victorian strain which is of very low virulence for fowls.

(4) No qualitative antigenic differences can be detected amongst these strains but the epizootic strains are more readily neutralised by immune serum than the enzootic Victorian strain.

The use of pock counting titration methods on the chorioallantoic membrane has not, so far as we are aware, been previously suggested. Guérin (8) made use of a method similar in principle for titrating vaccinia virus by counting the pocks produced after cutaneous application of the diluted virus to the shaved skin of the rabbit. Herzberg (9) titrated herpes virus by counting the number of focal lesions produced on the lightly scarified rabbit cornea with suitable virus dilutions and the same method has also been used by Bechold and Schlesinger (10). With those viruses such as laryngotracheitis virus for which it is suitable, the egg membrane technique appears to be the most convenient and accurate method of titration at present available, and it may possibly become the method of choice for quantitative work on virus problems of a general nature. The application of the method to other viruses is being investigated in this laboratory; vaccinia and infectious ectromelia of mice have given promising results.

The experiments on the nature of the virus-antiserum interaction show that with this virus at least there is an *in vitro* reaction between the two reagents which requires time to reach an equilibrium and which renders the virus particles unable to pass through a membrane filter. These two facts in themselves are sufficient to show that Sabin's contention that there is no interaction *in vitro* between antibody and virus particle is not applicable to the virus of laryngotracheitis and hence cannot be generalised to cover virus-antibody reactions in general. Three other facts bring the neutralisation of laryngotracheitis virus by immune serum completely into line with the phage-antiphage reactions as investigated by Andrewes and Elford. These are: (*a*) its adherence to the percentage law that with a given concentration of antiserum the degree of inactivation is independent of the concentration of virus; (*b*) reactivation by dilution is readily demonstrable, and (*c*) the average size of focus is much reduced with partially inactivated preparations.

Any theoretical discussion of the nature of the reaction in this case must necessarily be along the same lines as were followed by Andrewes and Elford in their work on bacteriophage. The hypothesis they put forward of an incomplete coating of antibody globulin on the phage particle seems to be readily enough applicable to the present reaction and probably to many other virus-antibody reactions. If the effec-

tiveness of a given degree of coating to prevent infection by a virus particle varies from one susceptible tissue to another, and if the degree of coating with antibody molecules is wholly or in part determined by a reversible reaction depending on the concentration of free antibody molecules, a reasonable interpretation of the phenomena is available which is quite harmonious with current views in other fields of immunology (Marrack (11)).

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EXPLANATION OF PLATE 46

FIG. 1. Photograph of egg membrane lesions produced by the virus of infectious laryngotracheitis.

a, stock suspension diluted 1:10 with normal fowl serum;
b, " " " 1:100 " " " "
c, " " " 1:1000 " " " "

d, *e*, *f*, serum 326 plus undiluted virus to show the influence of partial neutralisation by serum on the appearance of the egg membrane lesions.

(5) A study of the inactivation of the virus by immune serum shows that (*a*) the process of inactivation requires time for its completion *in vitro*; (*b*) the proportionate reduction in titre produced by a given concentration of antiserum is independent of the initial virus concentration; (*c*) reactivation by dilution is readily demonstrable; (*d*) virus in the presence of small concentrations of immune serum producing only a slight inactivating effect is rendered incapable of passing a gradocol membrane normally permeable to it; (*e*) the foci produced from partially neutralised virus suspensions are smaller than normal, suggesting delay in the initiation of foci.

(6) These findings bring the neutralisation of a typical virus by immune serum completely into line with the phage-antiphage reaction as described by Andrewes and Elford.

I am particularly indebted to Dr. H. R. Seddon, Director of Veterinary Research, New South Wales, for suggesting the application of the developing egg technique to the study of the current epizootics of infectious laryngotracheitis, for a liberal supply of virus material and immune sera and for testing the infectivity of egg virus material. I have also had the benefit of the coöperation of Dr. H. E. Albiston, Director of the Veterinary Research Institute of the University of Melbourne, of the Victorian Department of Agriculture and of Dame Jean Connor in obtaining material in Victoria.

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FIG. 1

(Burnet: Virus of infectious laryngotracheitis)

STUDIES IN SYNERGY*

The Synergic Action of Staphylotoxin and Beef Lens Extract in Rabbits

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The following experiments were stimulated by our interest in the subject of "allergic irritability" (1) and in the work of Burky (2) who observed that weakly antigenic substances, for example, beef lens and ragweed pollen, provoked in rabbits marked antibody formation and hypersensitiveness when combined with staphylotoxin produced by growing staphylococci in broth containing the weak antigen. He hypothesized that this was due to the conjugation, *in vitro*, of the poor antigen with the powerful antigenic toxin. Of particular interest was the marked sensitivity of rabbits to simple mechanical trauma after several injections of rabbit muscle toxin. Because we had observed comparable phenomena in rabbits treated in other ways we felt that the hyperreactivity might be due to factors arising in the animal's body rather than to those occurring simply *in vitro*. The present communication deals with tests of this hypothesis.

EXPERIMENTAL

The experiments, in general, were arranged to permit comparison of groups of animals injected in different ways with the two antigenic substances.

The rabbits were adults, of either an English-lilac-Havana hybrid stock, or pure Havanas, strains usually quite resistant to spontaneous infection. In any given experiment a single variety was employed, and usually animals were of the same sex. Only those with large areas of good skin were selected; the hair was removed with a fine mechanical clipper; and care was taken not to inject sites

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lens weighed 0.7 gm. It was ground to a fine powder with a sterile glass rod; 20 cc. of satisfactory broth was added; the medium was incubated 4 days to insure sterility, then was inoculated and treated in the same manner as the whole lens toxin.

Lens extracts were likewise prepared in two ways.

1. Extracted with Ammonium Hydrate as Recommended by Burky (2).—Fresh lenses were minced finely with scissors, then ground with sterile alundum, emulsified with N/100 ammonium hydrate containing 0.5 per cent tricresol and filtered through a No. V Berkefeld filter. The filtrate was adjusted with sterile normal saline solution so that it contained 2 per cent protein, then was stored in the refrigerator in a number of tubes.

2. Ringer's Solution Extracts of Frozen and Dried Lenses.—Lens extracts in ammonium hydroxide solution soon became turbid and induced increasingly larger lesions in the skins of normal rabbits. Because bacterial contamination was excluded, it appeared probable that the lens substance in this form of extract was subject to continuous autolysis effected by the enzymes which have been demonstrated therein. It therefore seemed obvious that such extracts would not be uniform because their properties varied with age, and hence their use would render valueless comparative observations made over a period of time. In order to obviate these objections the following method was used in all experiments except the first. Beef and rabbit eyes were secured soon after the animals were slaughtered; the muscles were removed and the sclera and cornea were covered with tincture of iodine, washed with alcohol and dried with ether. A transverse incision was made in the cornea with a sterile safety razor, and the lenses were "popped" into sterile tubes, and immediately frozen with CO_2 ice. After being dried *in vacuo* over phosphorous pentoxide while still frozen (5) the tubes were quickly stoppered with melted sealing wax. In this dried form the lenses could be kept for months without undergoing any autolytic disintegration. For preparing an extract a dried lens was ground to a fine powder in a sterile mortar, and then dissolved in Ringer's solution by slowly adding the solvent in much the same manner as when starch is put in solution. With ten volumes of solvent, computing the water equivalent of weight of the dried lens as one volume, at least 90 per cent of the lens dissolved.[2] It was centrifuged 10 minutes at moderate speed, and 1 cc. quantities of the supernatant opalescent liquid were pipetted into a number of tubes which were immediately frozen in CO_2 ice and subsequently kept frozen in a thermos jar containing this ice. The extracts were not melted until just before use. These precautions were considered necessary in order to prevent autolytic changes. That different extracts thus prepared were fairly uniform in content of lens protein was shown by the quantitative similarity of

[2] On the average a dried beef lens weighed 0.7 gm., and a dried rabbit lens 0.17 gm. The extracts were considered as 10 per cent solutions; and 0.1 cc. induced only evanescent erythema when injected into normal rabbit skins.

where coarse hair rapidly regrew, for Burky (3) has shown that skin bearing such a coat reacts differently to staphylotoxin than does that remaining hairless long after depilation.

The accessory substance used to induce reactions was staphylotoxin, made by growing *Staphylococcus aureus*, strain Ha,[1] for 10 days in beef heart infusion broth containing 1 per cent neopeptone and 0.5 per cent NaCl, reaction pH 7.8; to this 0.2 per cent dextrose was added several days before inoculation. In this medium the cocci grew readily, often in the form of a film on the surface of the broth and in a heavy sediment. The flasks containing the growing cocci were capped with lead foil held tightly in place with rubber bands, a procedure that probably induced partial anaerobiosis. After 10 days' incubation at 37°C. the supernatant clear medium resulting from centrifugation was pipetted into a sterile flask; 50 cc. of this was passed through a No. V Berkefeld filter and discarded; the remainder was mixed with tricresol to make a final concentration of 0.5 per cent and filtered through the same filter. It was stored cold in small fractions which were placed in a number of pyrex glass tubes, plugged with cotton and capped with lead foil. Such toxin retained its original potency for at least 12 months. It is not improbable that, because of the long incubation, this toxin contained considerable toxoid. Subsequent comparison with a toxin prepared in semisolid agar according to Dolman's technique (4) showed it to have about one-sixteenth of the dermonecrotizing capacity of the latter.

Toxin thus prepared from one particular lot of broth was quite powerful, and when injected intracutaneously in doses of 0.01 cc. induced in most rabbits necrotic areas 10 to 15 mm. in diameter surrounded by red edematous rings; in many animals 0.005 cc. was sufficient to cause necrotic lesions 8 to 10 mm. in diameter. Several rabbits receiving 0.1 cc. intracutaneously for the first injection died after 24 to 48 hours, and postmortem showed distended intestines, with numerous hemorrhages, and fatty degeneration of the liver and other parenchymatous viscera. An equivalent dose intravenously led to a higher proportion of deaths. Other lots of medium produced less potent toxin, and doses five to ten times as large were necessary to induce the effects described; but throughout these experiments only the strongest toxin was used.

Lens toxin, so called, was made in two ways: (*a*) Lenses removed aseptically from fresh beef eyes were placed in individual, large, foil-capped tubes each containing 20 cc. of the broth above described. After 4 days' incubation to insure sterility they were inoculated with staphylococcus strain Ha and incubated for 10 days at 37°C. A very heavy growth resulted. The medium was restored to original volume with broth, then centrifuged to clearness, tricresolized, filtered and stored in the same manner as the simple toxin broth. (*b*) Fresh beef lenses were placed in individual tubes, frozen in a dry ice-alcohol mixture, and dried *in vacuo* over phosphorus pentoxide. On the average, the resulting desiccated

[1] We are indebted to Dr. E. L. Burky for this strain.

EXPERIMENTAL RESULTS

It was first necessary to show that lens toxin prepared in broth containing dried and frozen lens powder (method 2) would be as effective in sensitizing rabbits as that used by Burky (method 1).

Experiment 1.—Rabbits were grouped in threes; group A received 0.1 cc. of the whole lens toxin intracutaneously on the 1st, 8th, 15th and 22nd days; group B received the same doses of powdered lens toxin; group C, the same doses of whole lens broth without toxin, and group D, powdered lens broth without toxin. By the beginning of the 3rd week groups A and B were showing markedly increased cutaneous sensitivity, and the other two groups only slightly increased reactions. Tested on the 29th days with lens extract containing no toxin, the first two groups had large edematous cutaneous lesions with red or purple necrotic centers, while the controls had only moderately edematous erythematous areas lasting less than 48 hours. Both the ophthalmic sensitivity and the precipitin reactions were distinctly more marked in group B than in group A, and they were negative in the other two groups.

This experiment confirmed Burky's observation concerning the powerful sensitizing action of staphylotoxin lens broth, and also indicated that frozen and dried lens in broth containing toxin was even a better sensitizing reagent. With both forms, the period required for sensitization was distinctly shorter than that reported by Burky.

We next determined whether it was necessary to have the toxin and lens conjugated *in vitro* by a long period of incubation, and also whether it was necessary to have the lens in the form of lens broth in which considerable autolysis must have occurred during incubation.

Experiment 2.—Three preparations of different character containing beef lens substance in approximately the same concentration were given in five intracutaneous injections (totalling 0.4 cc. in each instance) over a period of 22 days to four corresponding groups of rabbits. The lens substance given group B was that present in the filtrate of a staphylococcus culture grown in lens broth, and was hypothetically conjugated with toxin. Groups D and E received toxin and lens extract separately; and because it was found in previous experiments that many animals succumbed to larger amounts, the dose of toxin was reduced to a total of 0.09 cc. or less than one-fourth of the quantity received by group B. The toxin appropriately diluted with normal salt solution, was injected in 0.1 cc. volumes intracutaneously, followed immediately by the introduction of lens material at the same site and in equivalent volume. Lens substance was given to group D in the form of lens broth, and to group E as Ringer's solution extract. In these two groups, therefore, toxin and lens were not brought into contact out-

action as precipitinogens when tested with anti-lens precipitating serum. It is true that they probably contained all the crystallins and not chiefly the alpha one, as do ammonium hydroxide extracts; but this seemed desirable where the sensitizing and immunizing capacity of whole lens was to be studied.

Precipitin Reactions.—The frozen extracts, considered as 1–10 dilutions, were further diluted with physiological saline solution by steps of ten, up to a final dilution of 10^{-6}, and 0.4 cc. of each was placed in its suitable position in a series of tubes, to each of which 0.2 cc. of serum was added; the tubes were well shaken, incubated 1 hour in the water bath at 37°C. and placed in the refrigerator overnight. The intensity of the reaction in each tube was recorded by a series of plus marks. Usually all the sera obtained in a given experiment were kept cold and tested simultaneously; but when this was not done several sera from the previous bleeding were introduced in order to give readings of comparative strength throughout.

Cutaneous sensitivity was tested by injecting the lens extracts or other substances intracutaneously into the areas of skin previously clipped, still hairless, and not injected before. A constant volume of 0.1 cc. was employed. The length, breadth and thickness of the lesions were measured with calipers for 2 or more days until recession began; the color, consistency and amount of necrosis were also recorded.

Ophthalmic sensitivity was tested with practically the same technique as that employed by Burky (2). The rabbits were anesthetized with intravenous injections of nembutal, 40 mg. per kilo body weight. An assistant retracted the upper lid manually and pressed at the base of the lower one with a flat, blunt, instrument. The eyeball was thus thrown forward and fixed without being traumatized with toothed forceps. A very sharp No. 27 gauge needle attached to a tuberculin syringe was inserted through the middle of the cornea into the anterior chamber, and 0.1 cc. of aqueous humor was delivered into the syringe. The needle was then advanced well into the lens, and the aqueous in the syringe was slowly injected; finally, suction was applied by pulling on the plunger of the syringe to insure that the needle was not in the anterior chamber; then the needle was quickly withdrawn. Usually this procedure was followed by conjunctivitis lasting 2 or more days; and occasionally there was a line of rupture at the junction of the cornea and sclera the next day. Neither of these was considered evidence of specific sensitivity. The phaco-anaphylactic reactions, recorded as positive, consisted of increasing redness and edema of the palpebral and bulbar conjunctivae, corneal haze, often with vascularization, iritis, synechiae and marked lens opacities. These processes were usually increasing in intensity at the time the initial traumatic reactions in non-sensitive animals were receding. Occasionally there was a rupture of the optic globe in very sensitive animals during the period from the 2nd to the 4th days, probably due to high intraocular tension resulting from the severe inflammation. All tested eyes were observed at least 10 days, and longer when indicated. None was submitted to histologic examinations.

side the animal's body. Group G received lens broth only and was introduced to determine the sensitizing potentiality of such lens autolysate. The results are summarized in Table I.

The results showed that toxin and lens need not be conjugated *in vitro* in order to induce marked cutaneous and ophthalmic sensitivity, as well as marked precipitin formation. On the other hand, it apparently was necessary to have a combined action of toxin and lens in order to induce this high degree of reactivity, for when toxin was lacking (group G) the amount of cutaneous hypersensitivity induced was slight; there was no ophthalmic sensitivity, and no antibodies were present in the dilutions tested. It is noteworthy that the reduction in dose of toxin resulted in the development of an increased degree of hypersensitivity to lens substance, although the quantity of the latter injected remained constant. This is not surprising when the amount of focal necrosis and general intoxication following the two doses respectively is considered. In the large areas of focal necrosis many of the cells, which were in contact with toxin and lens, were killed; hence they were unable to participate in immune body formation. Marked general poisoning, moreover, would probably leave an animal less able to respond to another antigenic stimulus. That the response was to a lens element is indicated not only by the ophthalmic reactions but also by the cutaneous hypersensitivity to rabbit lens extracts, which, although less than those to beef lens extract, was still very marked. There were also marked precipitin reactions with rabbit lens extracts, an indication of the well known antigenic similarity of all mammalian lenses.

Although the results in Experiment 2 proved that conjugation of the two antigens outside the rabbit's body was not necessary in order to exert a marked synergic effect, the almost simultaneous injection of the two reagents into the same areas left open the possibility that such a conjugation might have occurred in the skin. Experiment 3 was devised to determine whether sensitization would occur when conjugation *in vivo* was less probable.

Experiment 3.—The staphylotoxin was the same as that previously used, but because the total period of treatment was a few days longer, a total quantity of 0.12 cc. was administered. One treatment was given the first week, and two per week the next 3. All the animals received the same doses of both toxin and

TABLE I

Comparative Immunizing and Sensitizing Influence of Staphylotoxin Combined with Beef Lens Broth or Beef Lens Extract in Cutaneous Foci

Group	1st to 22nd days: Immunized with — Staphylotoxin in form of		Beef lens in form of	Rabbit No.	27th day: Precipitin reactions 10^{-3}	10^{-4}	10^{-5}	29th day: Cutaneous reactions — Beef lens extract: Edema	Beef lens extract: Center	Rabbit lens extract: Edema	Rabbit lens extract: Center	31st day: Ophthalmic reactions
	cc.		*cc.*									
B	Toxin lens broth 2 x 0.05 3 x 0.1	Combined *in vitro* in toxin lens broth	Toxin lens broth 2 x 0.05 3 x 0.1	5-5	±	+	−	65 x 55 x 3	R 8 x 8	23 x 21 x 2	−	−
				5-6	+++	+++	−	50 x 35 x 3	P 40 x 20	57 x 45 x 2	P 24 x 20	+++
				5-7	++	++	−	70 x 55 x 5	P 18 x 13	40 x 35 x 3	P 18 x 16	++++
D	Toxin broth 2 x 0.01 2 x 0.02 1 x 0.03		Lens broth 2 x 0.05 3 x 0.1	6-1	++	++	+	95 x 60 x 5	P 13 x 10	85 x 60 x 4	R 25 x 20	+±
				7-3	++	+++	+	70 x 60 x 4	R 15 x 10	38 x 28 x 2	−	+++
				6-3	+++	++	+	75 x 58 x 5	P 40 x 16	80 x 70 x 5	P 20 x 20	−
E	Toxin broth 2 x 0.01 2 x 0.02 1 x 0.03		Lens extract 2 x 0.05 3 x 0.01	7-4	++++	++++	+	75 x 60 x 5	P 27 x 25	45 x 34 x 3	R 24 x 24	+++
				6-5	++	++	±	85 x 60 x 5	P 11 x 7	45 x 40 x 2	R 11 x 8	? (1)
				6-6	+	+	−	75 x 55 x 4	P 16 x 8	55 x 45 x 3	P 18 x 12	+
G	0		Lens broth 2 x 0.05 3 x 0.1	7-5	−	−	−	Faint pink	−	22 x 22 x 1	−	±?
				7-1	−	−	−	50 x 47 x 2	−	22 x 22 x 1	−	−
				7-2	−	−	−	20 x 20 x 1	−	23 x 23 x 1	−	? (1)

(1) indicates eye ruptured 1st day.

Unless otherwise stated the precipitin reactions indicated in the tables were those tested with beef lens extracts.

In all instances comparative reactions were tested with rabbit lens extract.

All measurements of cutaneous reactions recorded in millimeters.

R indicates red centers, not necrotic. P indicates purple necrotic centers.

beef lens extract intradermally; the differences consisted merely in variations in the mode of combining two reagents. In group A the lens extract was given intradermally into the same foci immediately after the toxin was injected; it thus duplicated the technique employed in group E of Experiment 2. In groups B and C the same areas of skin likewise received the two substances: in group B the lens extract was injected 3 hours after the toxin, and in group C the interval was 24 hours. In group D, on the other hand, although the injections were almost simultaneous, the toxin was injected on the right side and the lens on the left. All the rabbits received a test dose of 0.1 cc. of lens extract on the first day and only two showed very slight reactions. As different samples of the same frozen extract were used for the entire experiment it was believed that any increase in reactivity would indicate increased sensitivity. The essential results are summarized in Table II.

Although all the rabbits became more sensitive to lens, groups C and D showed simply cutaneous hyperreactivity, while groups A and B developed ophthalmitis phaco-anaphylactica when their own lenses were traumatized. Differences in these four groups respectively were also reflected in the intensity of formation of precipitins to lens extracts; reactions with beef lens extracts were only slightly more marked than were those with rabbit lens. Because in other experiments rabbits receiving the same amounts of beef lens extract without synergic irritants showed both smaller cutaneous sensitivity and weaker precipitins than did the last two groups, it may be assumed that the toxin given to the animals in this experiment had a distinct influence in increasing their reactivity to lens. Injected into the same foci after an interval of 3 hours, the two substances induced the same high degree of reactivity as when injected almost simultaneously. The fact that the administration of the toxin on one side increased the action of the lens which was injected on the other, indicates that stimulating influence was probably exerted systemically as well as focally.

In order to determine whether the focal reactions were necessary to induce high degrees of sensitivity and immune body formation, rabbits were given the toxin and lens extract intravenously at the same time and in practically the same doses as in Experiment 3. Toxin administered intravenously proved much more depressing and lethal than when given intracutaneously. Nevertheless, enough animals survived to indicate that the intravenous route of administration of

TABLE II

Influence of Varying Combinations of Staphylotoxin and Beef Lens Extract

1st to 25th days						15th day			27th day			29th day		31st day
Group	Immunizing intracutaneous injections				Rabbit No.	Precipitin reactions						Cutaneous reactions Beef lens extract		Ophthalmic reactions
	Staphylo-toxin	Beef lens extract	Area	Inter-val		10^{-3}	10^{-4}	10^{-5}	10^{-3}	10^{-4}	10^{-5}	Edema	Center	
	cc.	*cc.*		*hrs.*										
A	2 x 0.01	7 x 0.1	Same foci	0	2-6	+	++	±	+++	+++	+	50 x 45 x 3	Mottled	−
	5 x 0.02				3-4	+±	+±	±	+++	++	+	50 x 40 x 4	P 23 x 17	++++ (2)
					3-5	++	++±	+	++±	++±	+	50 x 44 x 4	P 10 x 8	++++
B	2 x 0.01	7 x 0.1	Same foci	3	3-6	+	+	−	+++	++±	+	40 x 40 x 4	P 8 x 8	++++ (7)
	5 x 0.02				3-7	+±	+±	+	+++	+++	+	60 x 45 x 4	P 12 x 12	++++
					3-9	+	+	±	++	++	+	55 x 45 x 3	Mottled	++++
C	2 x 0.01	7 x 0.1	Same foci	24	4-0	+	+	−	Dead 27th day					
	5 x 0.02				4-1	−	−	−	±	+	±	37 x 35 x 2	−	−
					4-2	−	−	−	+++	++	±	63 x 47 x 2	−	−
D	2 x 0.01	7 x 0.1	Different	0	4-3	−	−	−	+	±	−	28 x 26 x 2	−	Dead
	5 x 0.02				4-4	+	±	−	++	+±	−	37 x 37 x 3	−	? (1)
	Right	Left			4-5	−	−	−	+	−	−	35 x 35 x 2	−	−

(1), (2), (7) indicate that the respective eyes ruptured on day designated by numeral.

TABLE III

Effect of Injecting Staphylotoxin and Beef Lens Extract Intravenously, Both Combined and Separately, into Different Veins

Group	1st to 25th day: Staphylotoxin	1st to 25th day: Beef lens extract	Rabbit No.	15th day: Precipitin reactions 10^{-3}	15th day: Precipitin reactions 10^{-4}	15th day: Precipitin reactions 10^{-5}	29th day: Precipitin reactions 10^{-3}	29th day: Precipitin reactions 10^{-4}	29th day: Precipitin reactions 10^{-5}	29th day: Cutaneous reactions: Beef lens extract: Edema	29th day: Cutaneous reactions: Beef lens extract: Center	29th day: Cutaneous reactions: Rabbit lens extract: Edema	29th day: Cutaneous reactions: Rabbit lens extract: Center	Ophthalmic reaction 30th day
	cc.	*cc.*												
A	1 x 0.005 3 x 0.01 2 x 0.015 2 x 0.02	8 x 0.1 Mixed before injecting	2	+±	+±	−	++++	+++	+±	55 x 47 x 5	R 8 x 8	34 x 30 x 3	−	++++
			5	+	−	−	+±	+	−	18 x 13 x 1	−	12 x 12 x P	−	−
			7	+±	+±	−	++++	+++	+±	50 x 45 x 4	P 15 x 12	28 x 22 x 2	−	+++
B	1 x 0.005 3 x 0.01 2 x 0.015 2 x 0.02 Right ear vein	8 x 0.1 Left ear vein	3	++++	+++	+	++++	+++	+±	95 x 37 x 2	P 28 x 25	30 x 28 x 2	P 20 x 20	−
			1-0	++++±	+++±	−	++++	+++±	+	50 x 40 x 2	R 10 x 10	34 x 30 x 1	−	+
			1-1	++++	+++	±	++++	+++	++	50 x 42 x 5	P 25 x 25	28 x 20 x 2	P 12 x 12	++++
C	None	8 x 0.1	6	+	−	−	+±	++	+	25 x 22 x 1	−	−	−	−
			8	±	−	−	+±	+	−	40 x 35 x 2	−	12 x 12 x P	−	−
			1-3	+	−	−	+++	++	−	20 x 20 x 1	−	18 x 18 x P	−	±?

the synergic agents led to as marked precipitin formation and ophthalmic sensitivity as that shown by group A of Experiment 3; but less cutaneous sensitivity occurred. These results were confirmed in other experiments, where, again, the death of a number of animals made comparative results inconclusive. In all these the toxin and lens extracts were mixed before being injected; hence the criticism might be offered that conjugated antigens may have been presented to the immune body producing cells. To answer further the question whether conjugation of the two components was necessary the following experiment was performed.

Experiment 4.—Three groups of animals were employed. The first two received toxin and beef lens extract intravenously; the third received, by the same route, lens extract but no toxin. Because the toxin in previous experiments had been so lethal in its effects the individual doses in the first 3 weeks of treatment were reduced; two injections were given each week for 4 weeks, with a total quantity of 0.105 cc. of toxin in this period. In group A the toxin and lens extracts were mixed immediately before injection. In group B the toxin was injected into the right aural vein and the lens extract into the left. Considering the degree of dilution that must have occurred in the blood it is obvious that the minimum of conjugation could have occurred in that medium. All animals survived the period of testing. The results are shown in Table III.

The synergic effect of staphylotoxin and beef lens was somewhat greater when they were injected separately into the veins of different ears than when they were mixed before injection. This is indicated by a distinctly stronger formation of precipitins by group B on the 15th day, and slightly stronger concentration of these antibodies on the 29th day, also by more marked cutaneous sensitivity to both beef and rabbit lens. The ophthalmic sensitivity was comparable in the two groups. While the intravenous injection of beef lens extract alone led to some degree of cutaneous hypersensitivity, a hyperreactive state could not be demonstrated to exist in the interior of the eyes. The fact that the cutaneous reactivity to beef lens was much more marked than to that of rabbits, and also that the precipitins were distinctly stronger with beef lens, suggests that part of this sensitivity was due to a beef element.

The possibility of enhancing still further the hypersensitive state by increasing the dose of beef lens was next tested.

TABLE IV

Effect of Injecting Staphylotoxin and Beef Lens Extract Intracutaneously into Rabbits Actively Immune to Staphylotoxin

Day.....	1	8	11	18	22	24	Rabbit No.	15			29			29				30
								Precipitin reactions						Cutaneous reactions				Ophthalmic reactions
														Beef lens extract		Rabbit lens extract		
								10^{-3}	10^{-4}	10^{-5}	10^{-3}	10^{-4}	10^{-5}	Edema	Center	Edema	Center	
	cc.	cc.	cc.	cc.	cc.	cc.												
Beef lens extract	0.05	0.05	0.1	0.05	0.05	0.05	9-2	−	−	−	±	−	−	18 x 15 x 1	−	jv	−	−
Staphylotoxin	0.01	0.01	0.02	0.05	0.05	0.05	9-3	−	−	−	+	−	−	v, p	−	v, p	−	−
							9-5	−	−	−	+	−	−	55 x 40 x 1	−	jv	−	−

v, indicates visible; p, palpable; jv, just visible; jp, just palpable.

TABLE V

Effect of Neutralizing in Vitro Staphylotoxin, Made in Beef Lens Broth, with Antitoxin

Group	Day.....	1	8	11	18	22	24	Rabbit No.	15			29			29				30
									Precipitin reactions						Cutaneous reactions				Ophthalmic reactions
															Beef lens extract		Rabbit lens extract		
									10^{-3}	10^{-4}	10^{-5}	10^{-3}	10^{-4}	10^{-5}	Edema	Center	Edema	Center	
		cc.	cc.	cc.	cc.	cc.	cc.												
A	Beef lens broth staphylotoxin	0.05	0.05	0.1	0.05	0.05	0.05	2-2	++	++	−	+±	+±	+	110 x 70 x 5	P 25 x 15	45 x 40 x 3	R ±	++++
								2-3	+	+±	−	++	++±	+	110 x 80 x 10	P 35 x 18	40 x 40 x 2	R +	++++
	Antitoxin	0.05	0.05	0.1	0.05	0.05	0.05	2-4	±	+	−	++	++±	+	80 x 40 x 3	R 20 x 15	v, p	−	++
B	Beef lens broth staphylotoxin	0.05	0.05	0.1	0.05	0.05	0.05	2-5	+	++	±	+++	+++±	+±	100 x 65 x 6	P 42 x 30	65 x 50 x 5	P 20 x 20	++++
								2-6	+±	+±	−	++	++±	+±	100 x 90 x 8	P 14 x 8	35 x 35 x 3	R ±	±
	Normal rabbit serum	0.05	0.05	0.1	0.05	0.05	0.05	2-7	±	−	−	++±	+++	+±	90 x 60 x 3	P 20 x 15	jv	−	±?

Experiment 5.—A group of rabbits was immunized with intravenous injections of staphylotoxin in the same doses as in Experiment 4, group A, but mixed with 0.5 cc. of beef lens extract instead of 0.1 cc. As this experiment was contemporaneous with Experiment 4, the latter served as a satisfactory control.

The only essential difference observed was a stronger formation of precipitins on the 15th day in all animals receiving the larger doses. This difference had disappeared at the end of 4 weeks, when cutaneous and ophthalmic sensitivity were almost identical in the two groups. It seems probable, therefore, that a dose of not more than 0.1 cc. of beef lens extract was optimal for inducing the maximal hypersensitivity.

All evidence up to this point indicated that one effect of the toxin was to increase in some way the reactivity of the rabbits so that when they received the beef lens extract simultaneously they reacted rapidly to this relatively weak antigen. In order to test further the possibility that irritation from the toxin was the important element, the action of the two agents was tested in rabbits that had been made actively immune to toxin.

Experiment 6.—Three rabbits received repeated doses of staphylococcal toxin intracutaneously for 6 weeks. At the beginning of this period 0.01 cc. of toxin induced necrotic lesions about 10 x 15 mm. in diameter. At the end of the period 0.1 cc. induced no necrotic lesions, but there appeared in some of the rabbits an evanescent erythema which was probably due to some material in the broth. During the next 4 weeks the rabbits received a combination of toxin and beef lens extract intracutaneously twice a week. In numerous previous experiments it had been shown that this dosage rendered normal rabbits highly sensitive (see group E, Experiment 2, and group A, Experiment 3). Table IV shows the results in this group.

Table IV indicates clearly that rabbits actively immune to toxin respond in much the same manner to the combination of toxin and beef lens as do normal rabbits to the injection of the same lens alone. The active antitoxic immunity of the rabbits appeared to lower their responsive mechanism towards beef lens to the level of normal rabbits. This experiment, therefore, adds additional weight to the conception that the irritating effect of the toxin is probably an important element in heightening their response to the beef lens extract.

While the results of Experiment 6 were apparently so decisive, it

Attempts were, therefore, made to remove some of these technical objections.

Experiment 8.—The toxin was the simple broth toxin that had been used in most of the experiments. The antitoxic rabbit serum was similar to that employed in the previous experiment, but the proportion of antitoxin to toxin was fivefold that previously employed; and because the toxin dosage was reduced, that of the antitoxic serum was not increased. The beef lens was dissolved in Ringer's solution, and kept frozen until just before each injection. The toxin-antitoxin mixture, made up with normal saline to a volume of 0.1 cc. per dose, was incubated 4 hours at 37°C., and then injected into a marked site in the skin. ½ hour later the lens extract was injected into the same site. As controls, the members of group B were injected with the same doses of toxin mixed with normal rabbit serum, instead of the antitoxin, and those of group C were injected first with the normal serum followed by lens extract into the same sites. Group B was not entirely satisfactory because one member died 2 days after the first injection, and the other two members succumbed to the nembutal anesthesia on the 30th day. The cutaneous and precipitin reactions of this group were, however, satisfactory controls of group A; and we can be reasonably certain that at least one of them would have shown a positive ophthalmic reaction.

The results are in accord with those of the previous experiment: the antitoxin-neutralized toxin exerted the same powerful synergic action as the unneutralized, even though it was injected each time ½ hour before the lens extract. It is noteworthy that the ultimate result was induced with a total dosage of 0.16 cc. of neutralized toxin and 0.5 cc. of 10 per cent beef lens extract. Group C showed that this amount of lens extract injected into sites previously injected with normal serum had some antigenic effect, but this was relatively small when compared with the same antigen acting in a tissue and animal under the influence of staphylotoxin.

The fact that hypersensitivity developed when an interval elapsed between the time of injection of the two antigens would be an argument against any extracellular conjugation of the lens and toxin, for it appears that the cells of the tissues would probably have taken up most of the toxin before the lens was introduced. A phenomenon observed during the experimental procedure might possibly have had some bearing on the results. The mixture of toxin and antitoxin became very turbid while being incubated; and as microscopic examination and cultures of the mixtures always failed to reveal any bac-

was thought necessary to test the hypothesis further and determine whether the neutralization of the toxin before it was injected would remove its stimulating synergic influence. These experiments were performed in several different ways. Attempts were first made to neutralize the irritating action of the toxin contained in a lens broth—the same preparation that was used in group B of Experiment 2.

Experiment 7.—Beef lens staphylotoxin broth was mixed with equal parts of antistaphylotoxic serum that had been produced by prolonged intracutaneous and intravenous immunization of a rabbit with simple staphylotoxin. This serum in a dose of 0.0006 cc. neutralized the necrotizing action of 0.01 cc. of toxin; it is, therefore, evident that the amounts used in this experiment were ample to neutralize all immediate necrotizing toxic effects of the toxin contained in the lens broth toxin. The neutralized mixtures were given in the doses and intervals indicated in Table V, group A; and simultaneously group B received the same amounts of lens toxin broth mixed with normal rabbit serum. That the dermonecrotizing action of the toxin was quite effectively neutralized was indicated by the failure of the group A rabbits to show necrotic lesions following the first three injections, while all rabbits of group B had necrotic lesions 20 to 30 mm. in diameter. Because the rabbits in group B had developed large tracking edematous lesions following the third injection, it was thought best to test the reactivity of both groups to 0.1 cc. of simple lens extract solution on the 15th day. Much to our surprise, both groups had very large edematous lesions with purple or red centers—typical Arthus reactions. Because of the intensity of these responses, it was considered advisable on the 18th, 22nd and 24th days to reduce the dose of the lens toxin to that first used. The total dosage and reactions as usually tested are indicated in Table V.

A comparison of the reactions in the two groups makes it evident that the antitoxic serum had no inhibitory effect on the ultimate sensitizing and immune body inducing influence of the lens toxin than had the normal rabbit serum. If we postulate that the toxin and beef lens had formed an inseparable antigenic complex it might be possible to explain the results of the present experiment on the supposition that the antitoxin had had no effect upon this complex except to do away with the necrotizing action, leaving it free to act as an effective synergist. In this experiment, moreover, the technique employed made it necessary to inject the neutralized toxin and lens substance simultaneously, and, as previously mentioned, the lens must have existed partly in the form of an autolysate, which is known to exert more antigenic action than does unautolyzed tissue.

Attempts were, therefore, made to remove some of these technical objections.

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The results are in accord with those of the previous experiment: the antitoxin-neutralized toxin exerted the same powerful synergic action as the unneutralized, even though it was injected each time ½ hour before the lens extract. It is noteworthy that the ultimate result was induced with a total dosage of 0.16 cc. of neutralized toxin and 0.5 cc. of 10 per cent beef lens extract. Group C showed that this amount of lens extract injected into sites previously injected with normal serum had some antigenic effect, but this was relatively small when compared with the same antigen acting in a tissue and animal under the influence of staphylotoxin.

The fact that hypersensitivity developed when an interval elapsed between the time of injection of the two antigens would be an argument against any extracellular conjugation of the lens and toxin, for it appears that the cells of the tissues would probably have taken up most of the toxin before the lens was introduced. A phenomenon observed during the experimental procedure might possibly have had some bearing on the results. The mixture of toxin and antitoxin became very turbid while being incubated; and as microscopic examination and cultures of the mixtures always failed to reveal any bac-

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TABLE VII

Effect of Staphylotoxin-Antitoxin Mixtures Given Intravenously

1st to 25th days				15th day				25th day				29th day						31st day
Right ear			Left ear	Precipitin reactions								Cutaneous reactions						Ophthalmic reactions
Staphylotoxin	Antitoxin	Beef lens extract	Rabbit No.	10^{-3}	10^{-4}	10^{-5}	10^{-3}	10^{-3}	10^{-4}	10^{-5}	Beef lens edema: Edema	Beef lens edema: Center	Rabbit lens extract: Edema	Rabbit lens extract: Center	
cc.	cc.														
1 x 0.005	+ 0.01	8 x 0.1	4-6	+++	+++	+	+++	+++	+++	+	50 x 50 x 3	—	25 x 25 x 1	—	+++
5 x 0.01	+ 0.05		4-7	+++	++	±	+++	+++	++	+	60 x 40 x 5	P 10 x 8	30 x 30 x 2	—	0*
2 x 0.02	+ 0.1		4-8	+++	+++	+	+++	+++	+++	++	65 x 50 x 4	P 15 x 10	55 x 35 x 3	P 10 x 10	—

* Died from anesthesia.

TABLE VI

Effect of Staphylotoxin-Antitoxin Mixtures Given Intracutaneously

Group		Day	1	8	11	18	22	25	Rabbit No.	15 Precipitin reactions			29 Precipitin reactions			29 Cutaneous reactions: Beef lens extract		29 Cutaneous reactions: Rabbit lens extract		30 Ophthalmic reactions
										10^{-3}	10^{-4}	10^{-5}	10^{-3}	10^{-4}	10^{-5}	Edema	Center	Edema	Center	
			cc.	cc.	cc.	cc.	cc.	cc.												
A	Staphylotoxin mixed with Antitoxin		0.01	0.01	0.02	0.04	0.04	0.04	3-7	+±	++	+	++±	++	+	70 x 70 x 3	R 10 x 10	40 x 40 x 2	—	—
			0.05	0.05	0.1	0.2	0.2	0.2	3-8	++±	++±	+±	+++	++	+	75 x 75 x 5	P 20 x 15	55 x 45 x 4	R 8 x 6	±
	Beef lens extract		0.05	0.05	0.1	0.1	0.1	0.1	3-9	+++	+++	+±	+++±	+++	+±	80 x 60 x 5	P 15 x 15	55 x 40 x 2	P 10 x 8	+++
B	Staphylotoxin mixed with Normal rabbit serum		0.01	0.01	0.02	0.04	0.04	0.04	4-0	+++	+++	+	+++±	+++	+±	60 x 60 x 7	P 15 x 15	38 x 40 x 4	R 10 x 10	*
			0.05	0.05	0.1	0.2	0.2	0.2	4-1	....	....	....	....	....	....	....	....	....	....	†
									4-2	+±	++	±	+++	++±	+	30 x 35 x 6	P 15 x 11	25 x 25 x 3	R 10 x 8	*
	Beef lens extract		0.05	0.05	0.1	0.1	0.1	0.1												
C	Normal rabbit serum mixed with Saline		0.05	0.05	0.1	0.2	0.2	0.2	4-3	+	±	—	+±	+	—	50 x 50 x 5	?	jv, jp	—	—
			0.05	0.05	0.1	0.04	0.04	0.04	4-4	+	±	—	+	+±	±	40 x 40 x 3	—	22 x 20 x 1	—	±
									4-5	+	—	—	+	±	—	22 x 22 x 1	—	v, p	—	±?
	Beef lens extract		0.05	0.05	0.1	0.1	0.1	0.1												

* Died from anesthesia on 30th day.

† Died following first injection.

immune body production; and Lewis (8) noted that guinea pigs receiving such unbalanced mixtures had higher degrees of anaphylaxis when tested with horse serum than did controls receiving completely neutralized toxin. Ramon's (9) work, showing the possibility of enhancing the immunizing power of diphtheria or tetanus toxoid (anatoxin) by simultaneously injecting tapioca, calcium chloride or bacterial vaccines, is an example of combination immunity in which he attributes the enhanced antigenic action to the focal inflammation arising from the synergic substance; and he thinks that by retarding the absorption of toxin the inflammation increases its immunizing capacity.

Probably the most extensively studied example of "combination immunization" is the synergic action of a so called *Schlepper* with lipoidal partial antigens or haptens, a subject which had been extensively reviewed by Landsteiner (10). Landsteiner and Simms (11) observed that a purified Forssman antigen, *i.e.* an alcoholic extract of organs or cells, which had little if any immune body inducing capacity, became a very efficient antigen when injected in combination with pig serum; but in order to exert this additive function it was necessary that the two substances be mixed *in vitro* and injected together. Doerr and Hallauer (12) recorded that many sera of species heterologous to that of the species of animal being immunized exerted a similar synergic function, as did disrupted erythrocytes and non-pathogenic bacteria, both living and killed. Contaminated homologous sera had a comparable effect (13). It is perhaps of considerable import that in the studies of the *Schlepper* effect of various sera, swine serum has been shown to have the most powerful action, for it is well known that this serum has the most toxic action of any of those commonly used in immunological studies. Junghans (14) has shown that 2 cc. of pig serum injected subcutaneously into rabbits induces large edematous lesions, and also that repeated injections of small doses of this serum intravenously into hypersensitive animals induce much more valvulitis and arteritis than Vaubel (15) found in rabbits treated similarly with considerably larger doses of horse serum. Thus, the toxic properties of sera appear to go hand in hand with their ability to irritate mesenchymal cells.

There is considerable evidence that inflammatory foci can act as accessory factors to increase an animal's reactivity to antigenic agents. Moro (16) reported that by injecting tuberculin into areas along with vaccine virus or pig serum the subjects became sensitive to subsequent injections of tuberculin; and Dienes (17) states that by injecting horse serum or egg white into tuberculous foci of guinea pigs, the animals subsequently showed tuberculin-like responses to intracutaneous injections of the respective soluble protein. These animals also had much larger amounts of precipitins in their serum than did those treated with the horse serum or egg white alone. Hanks (18) repeated these observations, but found that if the tubercle bacilli were injected into one testis and a short time later horse serum were injected into the other, the guinea pigs subsequently gave tuberculin-like responses to the intracutaneous injections of horse serum. He thus suggested, from his observations, that the altered reactivity of the animals was due not only to the local inflammatory condition, but that a substance was possibly given

teria, the turbidity was attributed to an immune precipitate.[3] While this precipitate was no longer necrotizing, it might well have retained its capacity for stimulating the cells to heightened immune body production.

Finally, the effect of introducing neutralized toxin into the vein of one ear and the beef lens extract into that of the other was tested.

Experiment 9.—Samples of the same toxin and rabbit antitoxic serum used previously were mixed in the proportions shown in Table VII and incubated 4 hours in a total volume of 0.2 cc. A precipitate formed similar to that previously noted. This neutralized toxin was then injected intravenously in the right aural vein and 0.1 cc. of beef lens extract into the left. The animals showed no signs of intoxication, an indication that the poisonous effect of the toxin had been largely eliminated. The total dosage and summary of the tests are shown in Table VII.

This experiment confirms the two previous ones, in that the neutralized toxin had the same synergic stimulating effect as the unneutralized. It furthermore confirms the results indicated in Experiment 3, group B, that the two agents introduced into the blood stream in a manner calculated to prevent intimate mixing still had the same effect as when mixed *in vitro* and then injected. It thus furnished additional evidence strongly indicating that the toxin acts through some mechanism in the animal's body rather than by uniting directly with the lens and forming a new antigenic complex.

DISCUSSION

The data presented strongly suggest that we are dealing with an example of so called combination immunity, of which there are many examples in the literature.

Among the earliest observations were those of Rosenau and Anderson (6) with diphtheria toxin and horse serum antitoxin; shortly afterwards Gay and Southard (7) observed that when a mixture of these two substances contained an excess of toxin with a resulting larger focus of inflammation there was more marked

[3] It was subsequently determined that a similar precipitate was formed by mixing the same antitoxic serum with neopeptone broth. The immunization with toxin broth had thus apparently caused the rabbits to form immune bodies against some weakly, or partly, antigenic substance in the broth.

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of cells which are especially adapted to the production of antibodies. The process of inflammation is accompanied by the local stimulation of resting mesenchymal cells of a type that are considered as being active in forming antibodies. It seems logical to assume that the more of these cells there are available, the greater the subsequent antibody production. These cells might be rendered more active either directly by the stimulating power of the toxin, or indirectly by the poisoned tissue, for, as has often been suggested, this diseased tissue may act antigenically in an entirely different manner from normal tissue. Repeated insults doubtless act cumulatively so that cells far distant from the focus are more and more called into action. This was well illustrated by the enormously increased cutaneous sensitivity after the 10th to 14th days.

Finally, there is reason to believe that the stimulating action of the toxin was exerted not only locally but systemically, because the doses employed, although small, were close to those that not infrequently proved lethal to susceptible animals. Histological examination of their tissues showed a severe grade of poisoning, at times with early inflammatory reactions. Because many animals surviving the treatment had anorexia and diarrhea, as well as general signs of intoxication, it is probable that their tissues were irritated to a somewhat lesser degree. For this reason it seems likely that both focal and general factors played a part in the synergic effect of the two antigens.

SUMMARY

The degree of immunization and sensitization of rabbits following injections of beef lens is markedly increased when the animals are under the influence of staphylotoxin. Since the effect of the latter is exerted when the two substances are introduced separately into the same tissues with several hours elapsing between injections, or into different veins, it appears that an intimate association of them is unnecessary. A stimulating action of the toxin on the antibody-forming cells is a more probable explanation of the phenomena observed. Animals actively immune to staphylotoxin fail to show any synergic effect of this toxin when introduced with beef lens. Neutralization of the toxin *in vitro*, on the other hand, fails to eliminate this stimulating effect.

off from the focus that reconditioned the general reactivity of the animal. It is well known that tubercle bacilli are rapidly carried to other parts of the body when injected into tissues; hence it may be that these multiple areas first stimulated by the tubercle bacilli and then by absorbed horse serum were responsible, in part, for the reconditioning. Indeed Lewis and Loomis (19) several years earlier had shown that tuberculous animals respond more actively to other antigenic agents than do non-tuberculous controls, and named the altered state allergic irritability. We have discussed this important and interesting condition more fully elsewhere (1).

The rôle of certain metal salts (20, 21) as synergic agents in increasing the output of antibodies must also be considered in connection with the present problem. Schmidt's observations (22) indicate that the action of these salts is to cause a large outpouring of preformed antibodies from the immune body forming cells rather than to make these cells produce more immune substance. Glenny and his coworkers (23), on the other hand, have shown that diphtheria toxoid precipitated with potassium alum and other metallic precipitants (24) are more slowly eliminated from the tissues wherein they were injected; and thus the toxoid acts more continuously over a longer period. Hektoen and Welker (25) have demonstrated similar prolonged antigenic action of foreign proteins adsorbed on aluminum hydroxide.

One or more of the factors suggested by the foregoing incomplete review of related work must be considered in studying the results of the combined actions of beef lens and staphylotoxin, for there can be little doubt that the toxin increases the rabbit's response to beef lens. Burky's hypothesis that there has been an actual union of the toxin and lens into a new and more powerful antigenic complex would appear not to hold, in view of the fact that the two substances may be injected at different times into the same focus or even into the veins on the opposite sides of the body. Of course this does not insure a constant separation of the two, for doubtless both substances could be taken up by the same cells even though they were injected at different times, and under such circumstances they might conjugate *in vivo* intracellularly. Nevertheless, the synergic action of toxin and lens seems to be of a somewhat different nature from that of *Schlepper* and lipoid, for in the latter case some physical or chemical combination of the two agents outside the body is requisite.

The inflammatory focus might be considered an important element, both from the standpoint of impeding the rate at which the lens is fed into the body juices, and from that of a focal stimulation

STUDIES IN SYNERGY

Synergic Stimulating Effect of Hypersensitivity to Foreign Protein and to Bacteria

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Evidence has been presented in an accompanying paper (1) which indicates that the marked hypersensitivity of rabbits to lens extracts following injections of staphylotoxin and beef lens (2) is due to an enhanced stimulation of the animal's immune reactive mechanism by this toxin. In recent years numerous studies have indicated that comparable effects might accompany other synergic immunological states. In this connection we have investigated the conditioning influence of two types of so called allergy: (*a*) hypersensitivity from repeated inoculation with non-hemolytic streptococci, (*b*) hypersensitivity induced with a non-bacterial protein, horse serum. A report of these studies follows.

EXPERIMENTAL

The methods, in general, follow those previously described (1), especially the testing of the immune serum and of the hypersensitive response of the tissues to lens extracts. With certain exceptions the lens extracts were made in Ringer's solution from frozen and dried lenses. The non-hemolytic streptococci, strain Q 155, were known to be very good sensitizing agents when injected intracutaneously into rabbits. They were grown for 18 hours in a specially buffered broth (3) or in broth containing a few drops of rabbit blood; in one experiment lens broth was employed. In the latter case lenses were removed sterilely from the eyes, cut into eight pieces, four of which were placed in 10 cc. of broth. A number of tubes of media thus prepared were incubated for 2 days to insure sterility, then were covered with foil and stored in the refrigerator until needed. They were inoculated and used in the same way as the plain broth cultures, but probably contained more bacteria than did the latter, for the lens protein was a good enriching agent.

The first experiment was designed to determine whether combi-

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each week, so that the period of immunization covered 6 weeks. Tests made at various times are summarized in Table I.

Group E showed that the combination of indifferent streptococci and beef lens incubated together induced a fair degree of hypersensitivity to lens extract, and that this hypersensitivity extended to the interior of the animals' eyes. Group D, on the other hand, indicated that a similar state was induced when bacterial growth and lens extract were prepared separately and injected into the same foci in such a manner that conjugation of the two agents outside the animals' bodies did not occur. Comparison of groups D and C demonstrates that the synergic stimulating effect of the two reagents was more marked when they acted in the same foci than when they were introduced into opposite sides. The cutaneous hypersensitivity of group A to lens is noteworthy, and is an example of a state of allergic irritability induced by focal infection with streptococci. Animals in this state react to many different forms of injury (4, 5).

Although distinct hypersensitiveness to lens was induced by a combination of streptococcal infection and beef lens extract, this was not so intense as that observed in rabbits receiving staphylotoxin and lens. We have noted previously that it is possible to demonstrate the synergic influence of the toxin and lens when the two antigens are injected intravenously. It was therefore decided to compare the synergic stimulating influence of the non-hemolytic streptococci with that of staphylotoxin, both by intracutaneous and intravenous routes, with the same lot of beef lens extract. Because of the rapid development of marked hypersensitivity in the animals receiving the toxin, this group had one less treatment than did those infected with streptococci. The results are shown in Table II.

This comparative test indicates conclusively that the toxin was a more powerful stimulating agent than was infection with indifferent streptococci, but comparison of the various groups shows that the three reactions tested do not necessarily run parallel. In general, the rabbits treated intracutaneously with streptococci and lens had higher precipitin titers and more cutaneous sensitivity than did those immunized intravenously. Group A, treated with intracutaneous injections of streptococci and lens, developed as intense cutaneous sensitivity as did group BB that had received intravenous injections

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nations of streptococci and beef lens protein would sensitize rabbits to lens.

Two combinations were used: In group E (Table I) the bacteria were grown in beef lens broth, and used in a constant dose of 0.1 cc. of the medium. In group D the lens substance was in the form of an ammonium hydrate extract (2)

TABLE I

Comparative Immunizing and Sensitizing Influence of Indifferent Streptococci and Beef Lens Extract

Group	Day..... 1st to 43rd: Immunized with: Streptococcus Q-155	Immunized with: Beef lens extract	Rabbit No.	36th: Precipitins 10^{-1}	36th: Precipitins 10^{-2}	36th: Precipitins 10^{-3}	50th: Cutaneous reactions to beef lens extract: Edema	50th: Cutaneous reactions to beef lens extract: Red centers	52nd: Ophthalmic reactions
	cc.	cc.							
A	9 x 0.1	0	6-6	−	−	−	35 x 30 x 2	−	−
			6-7	−	−	−	42 x 40 x 2	−	−
			6-8	−	−	−	38 x 28 x v	−	(1)
B	0	9 x 0.1	6-9	−	−	−	26 x 22 x ½	−	−
			7-0	±	−	−	25 x 25 x ½	−	−
			7-2	+	±	−	8 x 8 x v	−	−
C	9 x 0.1 Right side	9 x 0.1 Left side	7-3	−	−	−	24 x 24 x v	−	−
			7-4	+	+	−	34 x 28 x 1	−	−
			7-5	+	±	−	18 x 18 x ½	−	−
D	3 x 0.1 1 x 0.01 5 x 0.001	Into same foci with 9 x 0.1	7-8	+++	++	±	41 x 40 x 4	18 x 18	−
			8-0	++	++	−	47 x 40 x 3	6 x 6	++
			8-1	+++	++	±	50 x 46 x 4	12 x 12	++
E	9 x 0.1	Grown in beef lens broth	8-3	+++	++	−	37 x 37 x 4	15 x 15	++
			8-4	+++	++	−	42 x 36 x 3	13 x 13	++
			8-7	+++	++	±	54 x 54 x 4	18 x 18	±

(1) indicates eye ruptured 1st day.

injected in a constant dosage of 0.1 cc.; the streptococci were grown in plain broth, and injected in decreasing dosage, because it had been previously shown that, as the rabbits become sensitive, it is necessary to inject smaller doses of these streptococci if one wishes to avoid inducing too much focal necrosis of the tissues. Three other groups of rabbits, shown in the table, served as controls. The first five injections were given at biweekly intervals and the next four once

of staphylotoxin and lens; the latter group, however, had marked ocular sensitivity, but only slightly stronger precipitins in their serum than had group A. Had simply precipitin formation and cutaneous sensitivity been used as indices of synergic action, these two groups would have indicated a relatively similar stimulating capacity of staphylotoxin and streptococcal infection; but the ocular reactions clearly differentiated them. On the other hand, with the exception of rabbit 3-0, group B, both parts of the experiment indicated that rabbits with focal lesions, induced by injections of lens extracts with bacterial agent, either living streptococci or staphylotoxin, were more strongly sensitized than were animals which received these materials intravenously. This suggests that some factor in the focus played a part in the eventual outcome.

Another way of inducing a hypersensitive state in rabbits is with a foreign, non-bacterial protein such as horse serum. There is experimental evidence indicating that small amounts of horse serum injected intracutaneously may induce a profound change in a rabbit's reactivity to non-related substances (4, 6). Riehm (7), furthermore, claims that a foreign protein, acting on a certain portion of one eye, tends to sensitize the corresponding part of the other eye. We therefore attempted to determine whether beef lens extract acted upon in a focus of non-bacterially induced hyperergic inflammation, would be a more effective sensitizing agent than when injected into normal tissue.

Three groups of rabbits, A, B and C, (see Table III) were injected each with 0.2 cc. of horse serum intracutaneously; then their reactivity to 0.001 cc. of the same serum was tested on the 8th and 11th days, and to 0.005 cc. on the 15th day. During the next 4 weeks the animals received biweekly intracutaneous injections of horse serum and beef lens extract as shown in Table III. Two additional normal control groups were introduced to test the effect of injecting these substances in non-sensitized animals over the same period. The usual tests were made as in previous experiments, with the results shown in Table III.

The results were in accord with those previously observed, *viz.* the animals having areas of focal reaction—in this instance to a foreign protein—reacted more vigorously to injections of beef lens extract than did animals not so sensitized. There was, however, no significant difference between the reactivity of animals in which the

TABLE II

Comparative Synergic Influence of Indifferent Streptococci and Staphylolotoxin

Group	Streptococcus Q-155	Beef lens extract	Rabbit No.	1st to 25th day	15th day			27th day			29th day		31st day
					Precipitins						Cutaneous reactions to beef lens extract		Ophthalmic reactions
					10^{-3}	10^{-4}	10^{-5}	10^{-4}	10^{-5}	10^{-6}	Edema	Purple center	
A	*cc.*	*cc.*											
	Intracutaneously into same foci		3-3		++	++	−	++	++	−	40 x 35 x 3	−	−
	1 x 0.01		2-8		++	++	±	++	++	±	45 x 35 x 3	−	−
	7 x 0.1	8 x 0.1	2-9		+++	++	+	....	Dead	....	....	....	....
B	Intravenously												
	1 x 0.01		3-0		+++	++	−	+++	+++	+	40 x 35 x 3	−	−
	7 x 0.1	8 x 0.1	3-1		+	−	−	+	−	−	v, p	−	−
			3-2		−	−	−	±	−	−	v, p	−	−
AA	Staphylotoxin												
	Intracutaneously into same foci		2-6		++	−	++++	+++	±	50 x 45 x 3	Mottled	−	
	2 x 0.01		3-4		+++	+	++++	++++	+	50 x 40 x 4	23 x 17	++++	
	5 x 0.02	7 x 0.1	3-5		+++		++++	+++	±	50 x 44 x 4	10 x 8	++++	
BB	Intravenously												
	2 x 0.01		4-6		++	−	....	Dead	....	....	....	....	....
	5 x 0.02	7 x 0.1	4-7		++	−	++++	++	+	35 x 35 x 2	−	++++	
			4-8		++	−	++++	++	±	40 x 40 x 2	−	++++	

focal infections with bacteria, so the difference in the two reactive states is likewise reflected by the manner in which the animal reacts to repeated injections of a second, or synergic, antigenic substance, such as beef lens.

Sensitizing Power of Rabbit Lens Extracts.—All our experiments, so far recorded, were carried out with beef lens extract, a substance heterologous to the rabbit, but which contains proteins or other constituents having immunochemical properties common to lenses of all mammals. Since Uhlenhuth's observations (8), showing that lenses of various animals have common antigenic properties, most investigators have found it extremely difficult, if not impossible, to induce the formation of precipitins to lens by immunization with homologous lens extracts. Hektoen and Schulhof (9) reported partial success in animals previously immunized with heterologous lens substance. Woods and Burky (10) fractionated lens into alpha, beta and gamma crystallins, and were able to obtain precipitins by immunizing with the respective crystallins; but mixing the beta with the alpha crystallin inhibited the latter's capacity to be precipitated by immune serum. The Ringer's solution extracts used in our experiments doubtless contained all three crystallins; nevertheless it was thought advisable to test whether the heightened reactivity of the animals when treated with staphylotoxin or streptococci might not furnish a favorable experimental set up for the demonstration of homologously induced sensitivity, and incidentally precipitin formation to any lens extract.

The extracts were prepared from frozen and dried lenses of normal rabbits. The toxin was the same as that used in the experiments recorded in the previous paper (1). The rabbits were treated over a period of 60 days, with one injection the first week and two per week thereafter. Because of the steadily diminishing reactivity of the rabbits to the toxin, due to the development of antitoxic immunity, it was necessary to increase the doses of toxin during the last half of the treatment, in order to induce lesions with small areas of dermonecrosis. In the entire period a total of 1.44 cc. of toxin and 1.6 cc. of 10 per cent lens extract was given to each animal. Group A received the two reagents into the same foci, and group B received them on opposite sides. By this technique the cutaneous reactivity of the latter group to lens could be closely followed; but in order to determine this reactivity in group A it was necessary to make occasional tests with rabbit lens extract alone. In neither group was there noteworthy

lens and horse serum were injected together and those receiving the reagents on opposite sides. The degree of precipitin formation and cutaneous hypersensitivity was distinctly less than in the case of rabbits receiving staphylotoxin or streptococci together with the lens extract; and no distinct ophthalmic sensitivity was demonstrated. It is true that the focal reactions in the horse serum-sensitive animals

TABLE III

Effect of Previous Sensitization with Horse Serum on Rabbits' Subsequent Response to Treatment with Beef Lens Extract and Horse Serum

Day..... 1st to 29th				19th			33rd			33rd	
Group	Horse serum	Beef lens extract	Rabbit No.	Precipitins to beef lens extract						Cutaneous reaction to beef lens extract	Ophthalmic reactions
				10^{-2}	10^{-3}	10^{-4}	10^{-2}	10^{-3}	10^{-4}		
	cc.	cc.									
A Previously sensitized with horse serum	8 x 0.005 Same foci	8 x 0.1	5-5	±	++	+	+	++	++	20 x 20 x 1	−
			5-9	−	−	−	+	+	−	17 x 17 x ½	−
			6-3	+	++	+	+	++	++	30 x 30 x p	±
B	Right 8 x 0.005	Left 8 x 0.1	5-6	−	−	−	±	−	−	v, p	?
			6-0	±	++	++	±	++	++	45 x 30 x 2	±
			6-4	+	+	−	±	+	+	22 x 22 x 2	−
C	8 x 0.005	0	5-8	−	−	−	−	−	−	−	−
			6-1	−	−	−	−	−	−	jv	−
			6-6	−	−	−	−	−	−	jv	−
D Not previously sensitized	8 x 0.005 Same foci	8 x 0.1	7-0	−	−	−	−	−	−	18 x 18 x p	−
			7-1	−	−	−	±	+	−	jv	−
			7-2	±	±	−	±	+	+	28 x 25 x ½	±
E	0	8 x 0.1	6-7	−	−	−	−	−	−	−	−
			6-8	+	+	±	+	+±	±	±	−
			7-3	+	+	−	±	+	±	±	−

were less intense than in those receiving toxin or bacteria. The lesions were, nevertheless, from 40 to 60 mm. in diameter and 2 to 3 mm. thick, and persisted 2 to 3 days without developing necrotic centers. It is evident, therefore, that the amount of reacting tissue was by no means small. Just as repeated injections of an animal with foreign protein induce less severe local lesions than do repeated

became more marked; the irises were more congested and edematous, and the previous opacity of the lenses became more intense. In other words, there was a late development of ophthalmitis interna less intense than usually occurs when the rabbits are sensitized with toxin and beef lens. This seemed to indicate that the rabbits had been sensitized, to a certain degree, by the long course of treatment with toxin and rabbit lens. The occurrence of ophthalmic reactions in group A and not in group B points to a sensitizing influence of the two substances when acting in the same area of focal inflammation.

Simultaneously with Experiment 1 of the present paper a group of rabbits was treated with a combination of Streptococcus Q-155 and rabbit lens extract made by dissolving normal rabbit lenses in weak ammonium hydrate. Presumably this was largely alpha crystallin. These animals received 30 daily intracutaneous injections with these two substances; a total of 3 cc. of 10 per cent lens extract was used. The precipitin formation in two animals was of the same intensity as in those of Experiment 4; the cutaneous reactions were only slight, and one animal developed distinct ophthalmitis phaco-anaphylactica a week after the lens was traumatized. Here again, it was evident that homologous lens extract was a much less efficient sensitizing reagent than that from beef.

DISCUSSION

The foregoing experiments throw additional light upon the synergic action of hypersensitivity to two different antigenic stimuli. The use of lens extract as the second antigen offers peculiar experimental advantages, because common antigenic fractions exist in all mammalian lenses. This permits a comparison of the effect of introducing homologous and heterologous lens extracts into animals, the responsive state of which has been enhanced by the accompanying induced synergic condition. It also permits us to compare the relative amount of antibody production with the intensity of cutaneous and ophthalmic sensitivity. While these three reactions are often parallel in degree there are enough exceptions to this rule to support the conception that they are not necessarily interdependent phenomena. When, therefore, one is interested in the mechanism by which certain lesions are induced by bacteria or foreign proteins, it is not sufficient

hypersensitivity at any time. No tests were made with beef lens extract until the end of the experimental period, when the reaction of the animals to simple broth was also tested; for Burky has noted that rabbits undergoing long immunization with simple staphylotoxin broth sometimes become sensitive to certain constituents of the broth. The results are summarized in Table IV.

All of the rabbits' sera gave equivocal precipitin reactions with 1–100 dilutions of the lens extract, and none in higher dilutions. The cutaneous reactions with lens extracts—both beef and rabbit—were

TABLE IV

Weak Immunizing and Sensitizing Influence of Staphylotoxin and Rabbit Lens Extract

Day..... 1st to 60th				60th		64th			67th
Group	Immunized with		Rabbit No.	Precipitins		Cutaneous reactions			Ophthalmic reactions
	Staphylotoxin	Rabbit lens		10^{-2}	10^{-3}	Beef lens	Rabbit lens	Broth	
	cc.	*cc.*							
A	2 x 0.01	Into same	7-9	±	—	v, p	jv	—	(1)
	5 x 0.02	foci with							
	3 x 0.04	2 x 0.05	8-0	+	—	—	—	—	++ Delayed
	2 x 0.1	15 x 0.1							
	5 x 0.2		8-1	±	—	15 x 12 x p	—	—	+ Delayed
B	2 x 0.01	2 x 0.05	8-2	±	—	19 x 19 x 1	v	25 x 25 x ½	—
	5 x 0.02	15 x 0.1							
	3 x 0.04		8-3	±	—	12 x 12 x p	v	—	—
	2 x 0.1								
	5 x 0.2		8-4	±	—	—	v, p	—	—
	Right side	Left side							

(1) indicates eye ruptured 1st day.

likewise practically negative; certainly they bore little resemblance to those of rabbits sensitized with much less toxin and beef lens. For a few days following the needling of the lenses it seemed that the eyes were no more sensitive than those of normal animals; in fact, group B reacted normally. About a week after the injury, however, two of the rabbits of group A showed a distinct increase in the signs of inflammation in the traumatized eyes; conjunctivitis

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to study the antibody curve in the sera of the treated animals, but one must compare this with the responses of the tissues to local insults under different immunological circumstances.

In by far the majority of investigations on the effect of infection, or on immunization with foreign proteins, the experimental set up has been with relatively normal animals. Obviously this is necessary if the complicated problems of infection are to be analyzed. In many human diseases, on the other hand, the circumstances are not so simple, for allergic and synergic reconditionings of the tissues are at play, as has been pointed out by Vaughan (11) and others. The different stimulating action of staphylotoxin poisoning, non-hemolytic streptococcal hyperergy and anti-horse serum hypersensitivity, in conjunction with injections of lens protein, illustrates well how each state must be studied. No doubt the use of more virulent bacteria, or of such infectious agents as filterable viruses, and also of more toxic sera might induce still different grades of response. Knowledge of the complicated antigenic mosaic of many bacteria furnishes an additional stimulus to investigation of the reactivity of experimental animals in different ergic states, for doubtless the response of an animal to infections with whole bacteria is different from that of one to chemically modified bacterial fractions, or to combinations of these fractions. It seems expedient, therefore, to study further both complex, as well as simple, ergic states in order to obtain a better understanding of the pathogenesis of some, as yet, little understood diseases.

CONCLUSIONS

1. The relative synergic stimulating influence of anti-horse serum sensitivity, non-hemolytic streptococcal hyperergy and staphylotoxin intoxication have been determined in connection with rabbits' reaction to simultaneous injections of lens extracts. These three synergic states are increasingly active in the order named.

2. Heterologous lens extract is a much more powerful antigen than is homologous lens, even under conditions where the reactivity of the immunized animal has been much enhanced.

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CHEMICAL STUDIES ON BACTERIAL AGGLUTINATION

II. The Identity of Precipitin and Agglutinin*

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Although the unitarian theory of antibodies has gained general acceptance since its formulation by Zinsser and others (1), quantitative evidence for the identity of agglutinins and precipitins has been lacking. Zinsser (2) has attempted to reconcile mathematically the apparent discrepancy between the serum dilutions in which agglutination and precipitation occur and Jones (3) has shown that collodion particles coated with egg albumin are agglutinated by small quantities of anti-egg albumin serum. Antibody concentrates, purified by Felton and Bailey (4), have been found to contain precipitins, agglutinins, opsonins, and protective antibody, but quantitative data were lacking until after the writers' preliminary report (5).

The development of quantitative micro methods for the estimation of precipitins (6, 7) and agglutinins (8) has now made it possible to investigate the quantitative correspondence of precipitin and agglutinin.

Pneumococcus Type I specific polysaccharide (acetyl form) (9),[1] a Chamberland filtrate of an autolyzed 8 day Type I Pneumococcus culture, Pneumococcus

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[1] A preparation of Type I pneumococcus specific polysaccharide in which the use of heat was avoided (9*b*) precipitated the same amount of antibody from horse antibody solution B 78 as did the original broth filtrate or material prepared according to (9*a*).

sion used was 2 ml., containing about 0.44 mg. of bacterial nitrogen or approximately 44 billion organisms.[3] This amount of suspension, therefore, contained no more than 0.001 mg. of specific polysaccharide. Similarly with a larger quantity of the same suspension and the stronger serum H 610, 0.66 mg. of bacterial nitrogen or approximately 66 billion organisms contained less than 0.001 mg. of type specific substance (Table I).

TABLE I

Absorption of Agglutinins from Horse and Rabbit Antisera with Pneumococcus I R, III R, and III S Suspensions

Absorption No.	1.0 ml. H 610 horse serum	4.5 ml. H 53300 horse serum	1.0 ml. R 3484 rabbit serum		
	Agglutinin N removed by I R	Agglutinin N removed by III R*		Agglutinin N removed by	
				III R	III S
	mg.	*mg.*		*mg.*	*mg.*
1	0.16	0.14		0.098	0.176
2	0.00	0.08	Aliquot of 1st supernatant	0.07	0.022
3		0.06	Aliquot of 2nd supernatant	0.014	
4		0.06			
5		0.05	Total agglutinin N	0.182	0.198
6		0.05			
7		0.04			
		Absorption discontinued			

In absorptions 2 to 7 on serum H 53300 the entire supernatant from the preceding absorption was used.

* Centrifuged sediment from 2.0 ml. suspension. Agglutinin N determinations with III R and III S suspensions gave 0.56 and 0.54 mg. N per ml., respectively.

In order to remove the group specific antibody, 12 ml. of antibody solution B 76 were repeatedly absorbed with Pneumococcus I R organisms (about 3.5 mg. N per absorption). The pneumococci were first centrifuged, the supernatant was poured off, and the antibody solution was added to the bacterial sediment, taking care to obtain an even suspension. After 24 or 48 hours the

[3] The number of organisms was determined by comparison with a suspension of pneumococci standardized by counting. The numerical correspondence of bacterial nitrogen and the number of organisms is purely accidental.

I S (Dawson M (10)) and R (Dawson S) strains, and Type I antipneumococcus sera were chosen as the objects of study. Serum H 701, which was a combined Pneumococcus I, II antiserum, was precipitated with a slight excess of Type II polysaccharide in order to leave only the Type I anticarbohydrate (*cf.* 8). To make the system approach as nearly as possible that of a single type specific hapten and its homologous antibody, the other sera were absorbed with C polysaccharide and protein derived from Type I R pneumococci. These sera still contained considerable amounts of group specific antibody (5), but it was found possible to prepare solutions containing antibody only to the type specific carbohydrate by repeated absorption with large amounts of Pneumococcus I R suspension.

Before using the Pneumococcus I R strain it was necessary to know whether it contained residual traces of type specific carbohydrate since it was to be used both for the determination of anti-R in the sera, and for the removal of the anti-R by absorption of the sera. That the method used was applicable was shown by a similar study of a Type III R strain, in which specific polysaccharide was actually present.

EXPERIMENTAL

The precipitin determinations were carried out by addition of known amounts of Type I pneumococcus specific polysaccharide to accurately measured quantities of absorbed serum or antibody solution and determination of the specifically precipitated nitrogen after 48 hours at 0° on the washed precipitates by the micro Kjeldahl method (6, 7, 11). Agglutinin determinations were made similarly (8), using measured volumes of washed pneumococcus suspensions and estimating the increase in nitrogen content. Heat-killed suspensions were used for all analyses, but formalinized suspensions were also used in absorbing sera. All determinations were run in duplicate at 0° for 48 hours in order to remove antibody as completely as possible (11).

1. Absence of Type Specific Polysaccharide in a Pneumococcus I R Strain.—The Pneumococcus I R strain used[2] (strain I-192 R (12)) had been degraded from a virulent Type I S strain (strain I-230 S) which was also used in all agglutinations with S organisms. It was assumed that if the I R suspension contained type specific substance it should remove all of the antibody from a type specific antiserum in a series of absorptions. The data in Table II on sera H 610, H 701, and antibody solution B 76 show that no antibody was taken out after the initial absorption. Since the limits of error of the quantitative agglutinin method are about ± 0.02 mg. of nitrogen and it was also found that 0.01 mg. of specific polysaccharide removed about 0.20 mg. of antibody nitrogen from antibody solution B 76, 0.02 mg. of antibody nitrogen would thus correspond to 0.001 mg. of specific polysaccharide. The amount of I R (Dawson S) suspen-

[2] Kindly supplied by Dr. Martin H. Dawson. The pneumococci used in this investigation were grown by Mr. C. M. Soo Hoo.

TABLE II

Quantitative Comparison of Agglutinin and Precipitin in Type I Antipneumococcus Sera and Antibody Solutions

Laboratory designation of serum	Volume of serum or antibody solution used	Agglutinin N removed by Pn I	Strain used	Precipitin N found in supernatant	Remaining antibody N with Pn I S suspension	Total antibody N	Antibody N per ml.	Volume of serum or antibody solution used	Precipitin N removed	Agglutinin N in supernatant	Strain used	Remaining antibody N removed by Pn I S suspension	Total antibody N	Antibody N per ml.
	ml.	*mg.*		*mg.*	*mg.*	*mg.*	*mg.*	*ml.*	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
Serum H 610	0.50	0.77*	I S	0.00	0.00	0.77	1.54	0.50	0.75†	0.05	I R		0.80	1.60
	0.50	0.32	I S	0.38	0.05	0.75	1.50	0.50	0.75†	0.02	I R	0.00	0.77	1.54
	0.50	0.25	I S	0.50	0.06	0.81	1.62	0.50	0.52††	0.26	I S		0.78	1.56
	0.50	0.16§	I R	0.59†	0.00	0.75	1.50	0.50	0.60	0.21	I S	0.01	0.82	1.64
Serum H 701 (1:1)**	1.00	1.16‖	I S	0.00	0.00	1.16	1.16	1.00	1.05	0.14	I S	0.00	1.19	1.19
	1.00	0.27	I S	0.73	0.16	1.16	1.16	1.00	1.05	0.13	I R	0.00	1.18	1.18
	1.00	0.33‖	I R	0.81†	0.03	1.17	1.17	1.00	0.89	0.31*	I S	0.00	1.20	1.20
Antibody B 76	0.50	0.82*	I S	0.00	0.00	0.82	1.64	0.50	0.75	0.01	I R	0.00	0.76	1.52
	0.50	0.53	I S	0.08	0.17	0.78	1.56	0.50	0.55	0.24	I S	0.00	0.79	1.58
	0.50	0.09§	I R	0.61†	0.07	0.77	1.54							
Antibody B 76 absorbed	0.50	0.61*	I S	0.00	0.00	0.61	1.22	0.50	0.52	0.10	I S	0.00	0.62	1.24
	0.50	0.16	I S	0.32	0.12*	0.60	1.20	0.50	0.54†	0.06	I S	0.00	0.60	1.20
	1.00	0.02	I R					0.50	0.35	0.25	I S	0.01	0.61	1.22
Antibody B 78 absorbed	1.00	0.69‖	I S	0.00		0.69	0.69	1.00	0.69†‡	0.05	I S	0.00	0.74	0.74
	1.00	0.30	I S	0.33	0.07	0.70	0.70	1.00	0.63	0.10	I S	0.00	0.73	0.73
								1.00	0.56	0.11	I S	0.02	0.69	0.69

* Two absorptions.

† Complete removal of anti-S as shown by excess S in supernatant.

†† 2 hours at 37°. Ice box overnight.

§ A second absorption with Pneumococcus I R removed no more antibody.

‖ Three absorptions.

** Contained 0.13 mg. anti-C N per ml.

‡ Broth culture filtrate of Pneumococcus I S used.

agglutinated bacteria were centrifuged off and the supernatant was poured onto another portion of centrifuged organisms. In this way undue dilution was avoided. After about 12 absorptions, a determination of the anti-R on 1.0 ml. samples of the supernatant showed 0.016 mg. N per ml., and since this was within the limit of error of the determination (8) and equalled only 1.3 per cent of the type specific anticarbohydrate still present, absorption was discontinued. In the preparation of a large quantity of serum or of Felton antibody solution (13) free from group specific antibody it is convenient to divide the material into 12 to 15 ml. portions and absorb repeatedly with I R organisms as described for B 76.

2. Presence of Type Specific Polysaccharide in a Pneumococcus III R Strain.—In a similar manner it was found that a III R strain[2] (M III R (12)) actually contained a small amount of Type III specific polysaccharide. It will be seen from Table I that on repeated absorption of a Type III S antiserum, H 53300, with the suspension, small amounts of antibody nitrogen were continually removed and a limit was not reached. On determining the total agglutinin in a smaller amount of a weaker serum, R 348$_4$, with both Type III S and R suspensions (Table I), it was found that the same total amount of antibody nitrogen was ultimately removed by both strains. These results were interpreted on the basis that the Type III R strain contained a small amount of specific polysaccharide and this was confirmed as follows:

To 5.0 ml. of the bacterial suspension in saline 2.6 ml. of normal sodium hydroxide were added and the mixture was allowed to stand at 37° for 72 hours. The solution was neutralized to phenol red with hydrochloric acid, and was made up to 25.0 ml. and centrifuged free from traces of insoluble material. The salt concentration was then about 0.9 per cent. 3 ml. of this solution were set up with 1.0 ml. of a calibrated antibody solution (14) and the suspension was found to contain 0.0154 mg. of type specific polysaccharide per mg. of bacterial nitrogen—an appreciable amount.

Two different lots of the Type I S suspension used were found by this method to contain 0.462 and 0.493 mg. of type specific polysaccharide per mg. of bacterial nitrogen. The analyses were made as in the case of the Type III suspension except that the Type I polysaccharide used in calibrating the serum was treated with alkali at 37° for 72 hours in the same way as the bacterial suspension of which the polysaccharide content was to be determined, since Avery and Goebel (9) have shown that alkali treatment decreases the precipitating power of the polysaccharide.

3. Identity of Agglutinin and Precipitin.—In Table II are given the precipitin and agglutinin determinations. As will be noted, total agglutinin and total precipitin estimations were run, and in other instances a portion of the precipitin or agglutinin was removed and the agglutinin or precipitin in the supernatant was determined. In the last column are given the values for total antibody found, either for total agglutinin or precipitin alone, or for the sum of the analytical values for agglutinin plus precipitin. Serum H 610 and antibody solution B 76 were the same as those used in the first paper of this series (8), except that

TABLE II

Quantitative Comparison of Agglutinin and Precipitin in Type I Antipneumococcus Sera and Antibody Solutions

Laboratory designation of serum	Volume of serum or antibody solution used	Agglutinin N removed by Pn I	Strain used	Precipitin N found in supernatant	Remaining antibody N with Pn I S suspension	Total antibody N	Antibody N per ml.	Volume of serum or antibody solution used	Precipitin N removed	Agglutinin N in supernatant	Strain used	Remaining antibody N removed by Pn I S suspension	Total antibody N	Antibody N per ml.
	ml.	*mg.*		*mg.*	*mg.*	*mg.*	*mg.*	*ml.*	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
Serum H 610	0.50	0.77*	I S	0.00	0.00	0.77	1.54	0.50	0.75†	0.05	I R	0.00	0.80	1.60
	0.50	0.32	I S	0.38	0.05	0.75	1.50	0.50	0.75†	0.02	I R		0.77	1.54
	0.50	0.25	I S	0.50	0.06	0.81	1.62	0.50	0.52††	0.26	I S		0.78	1.56
	0.50	0.16§	I R	0.59†	0.00	0.75	1.50	0.50	0.60	0.21	I S	0.01	0.82	1.64
Serum H 701 (1:1)**	1.00	1.16‖	I S	0.00	0.00	1.16	1.16	1.00	1.05	0.14	I S	0.00	1.19	1.19
	1.00	0.27	I S	0.73	0.16	1.16	1.16	1.00	1.05	0.13	I R	0.00	1.18	1.18
	1.00	0.33‖	I R	0.81†	0.03	1.17	1.17	1.00	0.89	0.31*	I S	0.00	1.20	1.20
Antibody B 76	0.50	0.82*	I S	0.00	0.00	0.82	1.64	0.50	0.75	0.01	I R	0.00	0.76	1.52
	0.50	0.53	I S	0.08	0.17	0.78	1.56	0.50	0.55	0.24	I S	0.00	0.79	1.58
	0.50	0.09§	I R	0.61†	0.07	0.77	1.54							
Antibody B 76 absorbed	0.50	0.61*	I S	0.00	0.00	0.61	1.22	0.50	0.52	0.10	I S	0.00	0.62	1.24
	0.50	0.16	I S	0.32	0.12*	0.60	1.20	0.50	0.54†	0.06	I S	0.00	0.60	1.20
	1.00	0.02	I R					0.50	0.35	0.25	I S	0.01	0.61	1.22
Antibody B 78 absorbed	1.00	0.69‖	I S	0.00				1.00	0.69†‡	0.05	I S	0.00	0.74	0.74
	1.00	0.30	I S	0.33	0.07	0.69	0.69	1.00	0.63	0.10	I S	0.00	0.73	0.73
						0.70	0.70	1.00	0.56	0.11	I S	0.02	0.69	0.69

* Two absorptions.

† Complete removal of anti-S as shown by excess S in supernatant.

†† 2 hours at 37°. Ice box overnight.

§ A second absorption with Pneumococcus I R removed no more antibody.

‖ Three absorptions.

** Contained 0.13 mg. anti-C N per ml.

‡ Broth culture filtrate of Pneumococcus I S used.

agglutinated bacteria were centrifuged off and the supernatant was poured onto another portion of centrifuged organisms. In this way undue dilution was avoided. After about 12 absorptions, a determination of the anti-R on 1.0 ml. samples of the supernatant showed 0.016 mg. N per ml., and since this was within the limit of error of the determination (8) and equalled only 1.3 per cent of the type specific anticarbohydrate still present, absorption was discontinued. In the preparation of a large quantity of serum or of Felton antibody solution (13) free from group specific antibody it is convenient to divide the material into 12 to 15 ml. portions and absorb repeatedly with I R organisms as described for B 76.

2. Presence of Type Specific Polysaccharide in a Pneumococcus III R Strain.—In a similar manner it was found that a III R strain[2] (M III R (12)) actually contained a small amount of Type III specific polysaccharide. It will be seen from Table I that on repeated absorption of a Type III S antiserum, H 53300, with the suspension, small amounts of antibody nitrogen were continually removed and a limit was not reached. On determining the total agglutinin in a smaller amount of a weaker serum, R 348_4, with both Type III S and R suspensions (Table I), it was found that the same total amount of antibody nitrogen was ultimately removed by both strains. These results were interpreted on the basis that the Type III R strain contained a small amount of specific polysaccharide and this was confirmed as follows:

To 5.0 ml. of the bacterial suspension in saline 2.6 ml. of normal sodium hydroxide were added and the mixture was allowed to stand at 37° for 72 hours. The solution was neutralized to phenol red with hydrochloric acid, and was made up to 25.0 ml. and centrifuged free from traces of insoluble material. The salt concentration was then about 0.9 per cent. 3 ml. of this solution were set up with 1.0 ml. of a calibrated antibody solution (14) and the suspension was found to contain 0.0154 mg. of type specific polysaccharide per mg. of bacterial nitrogen—an appreciable amount.

Two different lots of the Type I S suspension used were found by this method to contain 0.462 and 0.493 mg. of type specific polysaccharide per mg. of bacterial nitrogen. The analyses were made as in the case of the Type III suspension except that the Type I polysaccharide used in calibrating the serum was treated with alkali at 37° for 72 hours in the same way as the bacterial suspension of which the polysaccharide content was to be determined, since Avery and Goebel (9) have shown that alkali treatment decreases the precipitating power of the polysaccharide.

3. Identity of Agglutinin and Precipitin.—In Table II are given the precipitin and agglutinin determinations. As will be noted, total agglutinin and total precipitin estimations were run, and in other instances a portion of the precipitin or agglutinin was removed and the agglutinin or precipitin in the supernatant was determined. In the last column are given the values for total antibody found, either for total agglutinin or precipitin alone, or for the sum of the analytical values for agglutinin plus precipitin. Serum H 610 and antibody solution B 76 were the same as those used in the first paper of this series (8), except that

analytical method given should therefore be of service in indicating the completeness of dissociation and possibly the stability of variants.

Data regarding the identity of agglutinins and precipitins are summarized in Table II. In the Type I antipneumococcus horse sera H 610 and H 701 it will be seen that type specific precipitin plus the small amount of residual group specific agglutinin equals total agglutinin, and that total antibody nitrogen remains the same when only a portion of the type specific antibody (anticarbohydrate) is first removed either as agglutinin or precipitin and the remainder as precipitin or agglutinin. Since the value for total antibody nitrogen in the combined precipitin-agglutinin estimations is the result of three independent sets of analyses, each involving several successive determinations, the precision attained is not as great as in the estimation solely of total agglutinin or total precipitin. The difference between the extreme values in the various sera ranges from 3.5 to 8.5 per cent of the total antibody content.

The data for sera H 610 and H 701 indicate the quantitative correspondence of the type specific anticarbohydrate agglutinin and precipitin within the limits of accuracy of the method. Using the mean values for total antibody nitrogen, antibody solution B 76, both unabsorbed and absorbed, yielded about 11.5 per cent more agglutinin nitrogen than precipitin nitrogen, while solution B 78 showed only a 4 per cent excess of type specific agglutinin. Since serum H 610, from which solution B 76 was prepared, did not show this effect, it would appear that a portion of the antibody had been slightly altered in the process of purification, a possibility already considered by Felton (13).

While the data for total type specific agglutinin and precipitin show the same quantity of each form of antibody it would still be possible that different substances, present in equal quantity, were involved. This possibility is eliminated, however, by the experiments on the partial removal of precipitin and agglutinin (Table II), since such a reduction of the content of precipitin or agglutinin resulted in an equal reduction of the amount of agglutinin or precipitin, respectively, remaining in the solution. Evidence is thus given for the identity of the type specific anticarbohydrate agglutinin and precipitin in these sera.

B 76 was somewhat more concentrated. "B 76 absorbed" and B 78 are the antibody solutions from which practically all of the group specific antibody was removed as described above. H 701 was a New York City Department of Health Type I, II mixed antipneumococcus serum from which the Type II antibody had been removed. The data for antibody solution B 75 have already been presented in the preliminary note (5) and are therefore not repeated.

The Type I specific polysaccharide used contained a small amount of C substance (15), so that I R agglutinin values were smaller after removal of precipitin.

In the experiments on the partial removal of agglutinin and precipitin, a known volume of bacterial suspension (or of polysaccharide solution) was added to a measured volume of serum or antibody solution, and after removal and analysis of the precipitate (6–8), an aliquot portion of the supernatant was set up with another measured volume of polysaccharide solution (or bacterial suspension). Finally, an aliquot of this second supernatant was set up with a known volume of Pneumococcus I S suspension in order to remove any traces of antibody remaining. The values in the table are corrected for the aliquots taken.

DISCUSSION

In order to determine whether or not precipitin and agglutinin are identical it was desirable to limit the investigation as nearly as possible to a single type specific antigen or hapten and its homologous antibody. However, pneumococci contain group specific antigens, and antipneumococcus sera may show varying amounts of group specific as well as type specific antibodies. It was therefore essential to have available a strain free from type specific antigen both for the purpose of estimating quantitatively the group specific antibody in a serum and for absorption of group specific antibody from such sera. Owing to the large number of absorptions necessary, the presence of even small amounts of type specific polysaccharide would result in removal of much of the antibody it was desired to retain.

The application of the quantitative agglutinin method (8) to establish the presence or absence of an antigenic component in the R strains used is illustrated in the section on experiments and summarized in Table I. It may be noted that Dawson (16) found it possible to cause reversion of the Pneumococcus M III R strain (which contains specific polysaccharide) to the S variant by *in vitro* as well as *in vivo* methods, whereas the I-192 R strain (which is free from specific polysaccharide) was exceedingly stable and could only be converted into the S form by subcutaneous injection with I S vaccine. The

ACTIVE IMMUNIZATION OF GUINEA PIGS WITH THE VIRUS OF EQUINE ENCEPHALOMYELITIS

II. Immunization with Formolized Virus

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The virus of equine encephalomyelitis appears in the blood in the natural infection of horses before the appearance of clinical symptoms, and epidemiological and experimental findings favor the view that it is disseminated by insect vectors (1), especially the mosquito (2–4). Were immunization attempted by the use of active virus, the danger might arise of bringing about either lethal encephalitis, or blood infection in animals showing no clinical symptoms, thus producing in both instances suitable conditions for insect transmission of the disease. Hence an effective method of immunization without employing active virus would be clearly advantageous.

We regarded the possibility of immunizing animals with this virus, almost or completely inactivated by chemical means, with considerable skepticism in view of the number of failures thus far reported with other viruses. They had yielded results that were not hopeful, or at best inconclusive.

Early attempts were made with virus treated with phenol, glycerol containing phenol, chloroform, saponin, merthiolate, tannic acid, and aluminum hydroxide (5, 6). These procedures proved unsatisfactory because either inactivation could not be obtained or the antigenic powers of the treated virus were reduced below the desired degree. In our hands, however, the use of formalin gave more promise. After these studies were begun, an article appeared by Shahan and Giltner (7) in which was described the successful immunization of horses, both in the laboratory and in the field, with virus inactivated by 0.4 per cent formalin. Of over 400 horses, each given two doses of 25 cc. of the vaccine, none developed disease. After 6 to 16 weeks, of thir-

SUMMARY

1. The absolute, quantitative agglutinin method has been used for the determination of the presence or absence of small amounts of specific polysaccharide in pneumococcus variants.

2. A technique is described for the removal of group specific antibody from antipneumococcus horse serum.

3. The type specific anticarbohydrate agglutinin and precipitin are not only present in identical amounts in Type I antipneumococcus horse serum, but a reduction in one is also accompanied by a quantitatively identical reduction in the other, providing evidence for their actual identity. In purified antibody solutions somewhat more agglutinin than precipitin is found, possibly owing to alteration of a portion of the antibody in the process of purification.

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infected mouse brain per unit weight was found, as a rule, to possess greater infectivity after intracerebral inoculation of mice than guinea pig brain: the limiting infective dilution of the former being ordinarily 0.03 cc. of 10^{-7} to 10^{-8} and of guinea pig tissue 0.03 cc. of 10^{-6} (occasionally 10^{-7}) dilutions.

Preparation of Vaccine.—Infected mouse or guinea pig brain was ground with sterile sand and 0.85 per cent saline solution to a 20 per cent suspension. This was filtered through at least eight layers of sterile cotton gauze. A portion of the filtrate was removed for titration and to the remainder was added an equal volume of 0.8 to 2 per cent formalin in saline solution. The mixture was kept at room temperature for 1 to 4 days and then stored at 5°C. for 3 to 65 days until used. Before injecting the vaccine into animals, the formalin was neutralized by ammonium hydroxide against phenolphthalein indicator to avoid toxic reactions.

Procedure of Immunization.—Guinea pigs were injected subcutaneously twice at 5 to 9 days' interval with 1 cc. each time of the same vaccine. Exceptional dosages will be mentioned in the text. In order to obtain additional evidence of the immunizing capacity of the materials in another host, mice were injected intracerebrally (0.03 .cc.) and also intraperitoneally or subcutaneously (0.2 to 0.5 cc.), followed after 7 days by a similar intraperitoneal or subcutaneous dose.

Tests for Induced Immunity.—To obviate cross tissue reactions in tests for immunity, guinea pigs received only guinea pig passage virus and mice, mouse passage material. A suitable number of untreated control animals was included in each test and these were injected last to minimize virus deterioration. The immunity tests were given intracerebrally, intranasally, and subcutaneously. The intracerebral inoculation—more drastic, and containing, as a rule, 100 to 1,000 lethal doses—consisted of 0.15 cc. for guinea pigs and 0.03 cc. for mice. Desiccated virus was found to be preferable for this use since its potency could be better controlled. The intranasal test in guinea pigs consisted of an instillation of 0.05 cc. of freshly prepared 10 per cent virus in broth in each nostril, and the subcutaneous test of 1 cc. of this material. The nasal and cutaneous tests have been discussed in the first paper (5) and ordinarily represent at least 1 to 10 M.L.D.

Attempts to Find Active Virus in Formolized Preparations.—The determination, as completely as was possible, of the degree of inactivation of virus in the formalin preparations used for immunization was for the present problem of utmost importance. For one phase of the problem concerned the question of whether protection against experimental infection with formolized material was due to residual active virus or to "killed" virus. All available methods were therefore employed intensively in the search for active virus in the fifteen formol vaccines prepared and employed for immunization. The methods

teen vaccinated animals receiving an intralingual test dose that induced disease in all of thirteen controls, nine withstood the inoculation. Howitt, on the other hand (6), recorded inconclusive results in guinea pigs treated with 1 per cent formolized virus. We continued our investigations on formolized virus, and in view of the generally satisfactory outcome of Shahan and Giltner's earlier experiments (7), we adapted their method, with certain modifications, to our study.

In this paper we present experimental evidence to show first that it is possible to induce in guinea pigs and mice a high degree of resistance against infection by the use of formolized material in which the virus is inactivated, or reduced to an amount well below its ordinary minimal dose for effective immunization (5).[1] Secondly, we offer experiments on the mechanism of the induced immunity, especially with respect to the possible activity of any residual or undetected virus present in such preparations.

Materials and Methods

Virus.—The Eastern strain of equine encephalomyelitis (8), more highly invasive for guinea pigs and mice than the Western, was employed as fresh or glycerolated guinea pig or mouse brain.

Titration of Virus Infectivity.—In the first experiments the maximal infectivity of the virus sample before formolization was not determined. It was noted later that vaccines made in the same way but with virus from different sources manifested unequal immunizing powers. It was inferred that these differences might have been related to quantity of virus in the source material. In later preparations it was therefore titrated before adding formalin. To titrate, the method of intracerebral injection of anesthetized mice was employed as already described (5). A noteworthy point is that hormone broth pH 7.4 was used as diluent in titrations since repeated observations have shown that saline solution as a diluent reduces the end-point of activity of fresh or dried virus 10 to 100-fold. In addition,

[1] In the first paper of this series (5) it was found that 3,000 to 30,000 mouse infective units of untreated, active virus (m.i.u.) injected subcutaneously three times at weekly intervals are required to protect guinea pigs against 1,000 or more intracerebral lethal doses and one or two such injections are required to induce resistance against experimental infection by way of the nose or subcutis. Larger quantities, even when given less than three times, are also immunizing but then the incidence of fever, blood infection, and death during the period of immunization increases; and in proportion as the optimal dosage, as mentioned, is decreased, so is the degree of induced immunity diminished.

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Method 5.—Nine guinea pigs received 0.15 cc. intracerebrally and 1.5 cc. subcutaneously of a formol preparation concentrated fivefold by vacuum distillation and desiccation. One of the animals was killed on the 2nd, another on the 4th, and a third on the 6th day after treatment. In each case the organs as described in Method 3 were tested for the presence of virus by mouse injection.

Method 6.—Eight of the formol vaccines were diluted and over 160 mice were used in these tests.

These tests were considered to be fully sufficient to indicate the presence of virus in the formolized preparations, inasmuch as the quantities put to test were even larger than the immunizing dose given to animals. The vaccines, thoroughly studied by means of the described methods, showed that thirteen of fifteen failed to yield virus (Tables I and II). A description of the two materials which contained a barely detectable quantity of virus follows.

Vaccine V, undiluted and diluted 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}, was injected 5 days after its preparation in the brain and peritoneal cavity of a total of 60 mice. Only two of twenty mice receiving the undiluted, and one of ten the 10^{-1} dilution developed the lethal virus disease, although after a prolonged incubation period (7 to 8 days). The same vaccine, undiluted, tested 2 days later, showed no virus. With it eight guinea pigs were injected intracerebrally (these animals are equally as reactive as mice after such injection) and also intraperitoneally and subcutaneously with 1.5 to 8 cc. They were unaffected. Of three guinea pigs of the group just cited which received the 8 cc. dosage, one was sacrificed on the 3rd, a second on the 4th, and a third on the 7th day after inoculation, and by means of the mouse intracerebral test, no virus was recovered from their blood or tissues. In addition, three of the remaining five guinea pigs of this group were submitted to an intracerebral test of 1,000 M.L.D. 1 month after the primary inoculation and all survived. We believe that while meagre amounts of active virus were present in this vaccine (V) on the 5th and none on the 7th day after its preparation, yet the substance was highly antigenic. This induced resistance, brought about with what is apparently virus-free material, will be described later.

Vaccine XIII (Table II). Only one of eighteen mice receiving the undiluted vaccine in the brain and subcutis on the 5th day after its preparation succumbed to virus encephalitis; twenty mice given 10^{-1} and 10^{-2} dilutions remained well. It is of interest that of four guinea pigs first treated with this vaccine on the day the latter showed the minute amount of virus, all failed to resist from 1,000 to 3,000 M.L.D. injected cerebrally 77 days later. The latter observation confirms other data to be presented pointing to the immunizing action of formolized material as being due to some other factor than to residual active virus.

can be classified as follows: (1) Intracerebral inoculation of vaccines in mice and guinea pigs together with simultaneous intraperitoneal or subcutaneous injection of massive doses (up to 8 cc.). (2) Serial (three to four) passages in mouse and guinea pig brain of cerebral tissue[2] inoculated with vaccines. (3) Search for active virus in blood, skin at site of injection, regional lymph nodes, spleen, liver, lungs, testis or ovary, kidney, brain, and cord of animals treated as in 1. (4) Inoculation of tissue cultures with as much as 1 cc. of vaccines to each culture flask. This is a new method devised for the purpose of testing the same amount of the vaccine as is used in the animal and is more sensitive in detecting virus than is animal inoculation (9). (5) Concentration of vaccine fivefold by vacuum distillation and desiccation followed by Methods 1, 2, 3, and 4. (6) Dilution method (10): (*a*) direct dilution; (*b*) dilution and keeping at 37°C. for as long as 10 days followed by tests each day by Methods 1, 2, and 4.

The following examples are given of the numerous attempts made to recover active virus from the vaccines.

Method 1.—327 mice and forty guinea pigs received intracerebral and subcutaneous or intraperitoneal injections of undiluted ammonium hydroxide-neutralized vaccines—fourteen being employed in mice and seven in guinea pigs. Mice received 0.03 cc. in the brain and 0.2 to 0.5 cc. by the other routes, while guinea pigs were given 0.15 cc. intracerebrally and 1 to 8 cc. subcutaneously or intraperitoneally.

Method 2.—54 mice and six guinea pigs were employed in an effort to recover active virus from three of the formol preparations by serial passage in brain tissue, as outlined above.

Method 3.—Twenty-eight anesthetized guinea pigs receiving eleven different vaccines were bled from the heart on the 2nd to the 14th day after intracerebral and subcutaneous injection of vaccine, and each specimen so obtained was tested for virus by intracerebral injection of four mice. Twelve guinea pigs treated similarly with three formolized preparations were killed on the 2nd to 7th day after the vaccine injection and a total of 384 mice were used in the attempt to detect virus in the different organs mentioned, and in the blood. Each of the tissues was ground to an approximate 10 per cent suspension in hormone broth pH 7.6 and after centrifugation at 2,000 R.P.M. for 10 minutes, the supernatant fluid was injected into the brains of four mice.

Method 4.—59 tissue cultures were each inoculated with from 0.5 to 1 cc. of undiluted, formol-neutralized material derived from three different vaccines.

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[2] All such operations were made with the aid of full ether anesthesia.

IX	"	" "	0.5–24	"	5	2 sc, 1 cc. each, 7 d. int.	36	"	>1,000	8/8
IX	"	" "	0.5–24	"	5	2 " 1 " " 7 " "	22	"	>10<100	4/4
X	"	3×10^6	1.0–40	"	7	2 " 1 " " 7 " "	22	"	>1,000	4/4
XI	F M B 5°C. 8 d.	3×10^7	0.5–24	"	5	2 " 1 " " 7 " "	22	"	>1,000	5/5
XI	" "	3×10^7	0.5–24	"	12	2 " 1 " " 7 " "	92	"	>1,000	4/4
XI	" "	3×10^7	0.5–24	"	65	2 " 1 " " 7 " "	40	"	>1,000<5,000	5/5
XII	F M B 5°C. 17 d.	3×10^8	0.5–24	"	5	1 " 1 "	87	"	>1,000	2/3
XII	" "	3×10^9	0.5–24	"	5	1 " 2 "	87	"	>1,000	3/3
XII	" "	3×10^8	0.5–24	"	5	2 " 1 " each, 7 d. int.	80	"	>1,000	3/3
XIV	F M B 5°C. 19 d.	3×10^7	0.5–24	"	7	2 " 1 " " 7 " "	38	"	>1,000	5/5
XIV	" "	3×10^7	0.5–24	"	7	1 " 1 "	38	in	N T 0/4	3/4
XIV	" "	3×10^7	0.5–24	"	7	2 " 1 " each, 7 d. int.	31	"	" 0/4	4/4
XIV	" "	3×10^7	0.5–24	"	7	1 " 1 "	38	sc	" 0/4	4/4
XIV	" "	3×10^7	0.5–24	"	7	2 " 1 " each, 7 d. int.	31	"	" 0/4	4/4
XIV	" "	3×10^7	0.5–24	"	7	2 " 0.2 cc. each, 5 d. int.	31	"	" 2/8	4/5
XIV	" "	3×10^7	0.5–24	"	7	5 " 0.2 " " 5 " "	16	"	" 2/8	5/5
XV	F M B	3×10^7	0.5–24	"	19	2 sc, 1.0 cc. each. Also ic 0.15 cc. time of 1st sc	14	ic	>1,000	5/5
XV	"	3×10^7	0.5–24 17 d., later conc. 5-fold	"	19	2 sc, 1.5 cc. each. Also ic 0.15 cc. time of 1st sc	14	"	>1,000	5/5

* 2 sc, 1 cc. each, 9 d. int. = two subcutaneous injections, 1 cc. each at 9 day interval.

† N T 2/2 = not titrated, none of two control guinea pigs survived.

‡ An ic test of >1,000 M.L.D. given 167 days after the last immunizing dose revealed no resistance in either of two guinea pigs.

F M B, F G B = fresh mouse or guinea pig brain.

sc = subcutaneous. in = intranasal. ic = intracerebral.

TABLE I

Formolized Vaccines That Protected Guinea Pigs against Infection

No. of vaccine	Source of virus	M.l.u. per cc. before inactivation	Per cent formalin and hours of contact at 22°C.	Test for active virus	Immunization			Test for immunity		
					Interval between preparation and use	Route and dose	Interval between last injection and immunity test	Route of injection	M.L.D.	Result No. of survivors
			per cent hrs.		*days*		*days*			
I *a*	F M B	Not tested	0.4–24	Negative	4	2 sc, 1 cc. each, 9 d. int.*	12	ic	N T 0/2†	3/3
I *b*	"	"	0.4–48	"	4	2 " 1 " " 9 " "	12	"	" 0/2	2/2
I *c*	F G B	"	0.4–96	"	4	2 " 1 " " 9 " "	12	"	" 0/2	3/3
II	"	"	1.0–24	"	4	2 " 1 " " 9 " "	12	"	>1,000	3/3
II	F M B	"	1.0–24	"	4	2 " 1 " " 6 " "	12	"	>10<100	1/1
III	"	"	0.4–24	"	4	2 " 1 " " 6 " "	20	"	>1,000	2/3
IV			0.4–24	"	63	2 " 1 " " 6 " "	75‡	"	>1,000	3/3
V	"	"	Neutralized and desiccated 0.4–24	"	12	2 sc, 1.2 cc. each, 7 d. int. Also ic 0.15 cc. time of 1st sc	16	"		
VI	"	"	0.4–24	Positive 5th day Negative 7th day	7	2 sc, 1 cc. each, 6d. int. Also ic 0.15 cc. time of 1st sc	10	"		
VIII	F G B 5°C. 2 d.	"	0.4–24	Negative	16	Used to immunize mice. See Table V	30	"	>1,000	3/3
VIII	" "	"	0.4–24	"	4	2 sc, 1 cc. each, 7 d. int.	—	—	—	—
IX	F G B	"	0.4–24	"	4	2 " 1 " " 7 " "	23	in	N T 0/3	3/3
		"	0.5–24	"	5	1 sc, 5 cc. with ic of 0.15 cc.	23	sc	" 0/3	3/3
							29	ic	>10<100	3/3

TABLE II

Vaccines That Failed to Protect Guinea Pigs Uniformly against Infection

No. of vaccine	Preparation	M.i.u. per cc. before inactivation	Test for active virus	Immunization			Test for immunity		
				Route and dose	Interval between preparation and use	Interval between last injection and immunity test	Route of injection	M.L.D.	Result / No. of survivors
					days	*days*			
IV	F M B 0.4% formalin at 22°C. 24 hrs., 5°C. 5 days. Then neutralized with NH_4OH and desiccated over H_2SO_4	Not tested	Negative	2 sc, 1.2 cc. each, 7 d. int.	12	10	ic	>1,000	3/3
					12	37	"	>1,000	0/3
					33	15 to 24	"	>1,000	0/7
VII	Glycerolated mouse brain stored at 5°C. 20 days. 0.4% formalin 22°C. 24 hrs., 5°C. 6 days	" "	"	2 sc, 1 cc. each, 7 d. int.	6	16 to 25	"	>1,000	2/7
VIII	Guinea pig brain stored at 5°C. 48 hrs., 0.4% formalin 22°C. 24 hrs., 5°C. 4 days	" "	"	" "	4	23	in	N T 0/3	3/3
					4	23	sc	" 0/3	3/3
				1 sc, 1 cc. each	4	18 to 37	ic	>100	3/6
					4	25	"	>100	1/4
					4	30	in	N T 0/3	1/3
					4	30	sc	" 0/4	4/6
XIII	Glycerolated guinea pig brain stored at 5°C. 25 days. 0.4% formalin 22°C. 24 hrs., 5°C. 4 days	3×10^4	Positive on 5th day	2 sc, 1 cc. each, 7 d. int.	5	77	ic	>1,000-<3,000	0/4

Abbreviations as in Table 1

Thus two of fifteen formolized vaccines contained barely detectable amounts of virus; one was immunizing; the other not. The one which was antigenic was, interestingly enough, the vaccine that contained no detectable virus at the time of its use in preventive inoculation.

Immunizing Capacity of Formolized Preparations of Virus

It is clear from the foregoing section that the formalin treatment either wholly inactivates the virus or, in exceptional instances, reduces its quantity to a point at which it by itself is not the factor involved in inducing immunity, as was shown in the preceding paper (5). The question now is whether such material is effective as a vaccine for the production of resistance against infection with the usual test doses. The answer is to be found in the data listed in Table I.

Reference to the table reveals that protection was acquired by 103 of 107 guinea pigs receiving subcutaneous injections of the thirteen different samples of formolized vaccines in which virus could not be detected by the recorded methods. Moreover, the vaccines were kept in the cold up to 65 days and still possessed the capacity to immunize; and the period of acquired resistance endured for at least 92 days—the duration of protection over longer intervals will be described later. The test dose for induced immunity was given either intranasally, subcutaneously, or intracerebrally and in the latter instance consisted, as a rule, of 1,000 M.L.D. or more.

Two of the fifteen formolized preparations were wholly ineffective for immunization (vaccines VII and XIII, Table II) and two were only partially antigenic (vaccines IV and VIII, Tables I and II).

Vaccines VII and XIII were prepared from glycerolated material, and one of them (XIII) was already referred to as containing a minimum of active virus at the time of its use. The latter material showed a relatively low titre of 3×10^4 m.i.u. before formolization and the effect of glycerolation on reduction of antigenicity is illustrated by the following experiment.

Vaccine X (effective) was prepared from the fresh, left cerebral hemispheres derived from ten guinea pigs prostrate from the experimental disease, while vaccine XIII (ineffective) was made from the right hemispheres of the same animals but after storage in 50 per cent glycerol for 25 days at 5°C. Storage in glycerol diminished the virus activity in the tissue by about 100 fold.

No titrations were made with the materials entering into the composition of vaccine VII.

In both materials the formalin was not neutralized by ammonium hydroxide until just before animal inoculation. By way of contrast, vaccine IV, neutralized just after inactivation by formalin had been completed, rapidly lost its capacity to immunize (Table II).

Relation of Dose and Dilution of Vaccines to Induced Immunity.— The results thus far tabulated consistently show a relation of the dose

TABLE III

Duration of Immunity in Guinea Pigs against Intracerebral Infection

No. of vaccine	Source of virus	M.l.u. per cc. before inactivation	Test for active virus	Immunization			Test for immunity	
				Interval between preparation and use	Route and dose	Interval between last injection and immunity test	M.L.D.	Result / No. of survivors
				days		*days*		
II	F G B	Not tested	Negative	4	2 sc, 1 cc. each, 6 d. int.	75	>10<100	1/1*
XI	F M B 5°C. 8 d.	3×10^7	"	12	2 sc, 1 cc. each, 7 d. int.	92	>1,000	4/4
XII	F M B 5°C. 17 d.	3×10^8	"	5	1 sc, 1 cc.	87	>1,000	2/3
XII	" "	3×10^8	"	5	1 sc, 2 cc.	87	>1,000	3/3
XII	" "	3×10^8	"	5	2 sc, 1 cc. each, 7 d. int.	80	>1,000	3/3

Abbreviations as in Table I.

* Only one animal of a series survived intercurrent infections during the period prior to the immunity test.

of a vaccine to the immunity induced in guinea pigs. Consequently two injections, each of 1 cc., are practical for immunization. The relation of quantity is again brought out in experiments on mice, summarized in Table V. Undiluted preparations yielded complete, whereas 10^{-1} dilution, little or no protection, and still greater dilutions none.

In vaccine IV the usual technique was modified without good results (Table II), while vaccine VIII was only partially effective; its virus content was not determined prior to formolization.

It would appear, therefore, that the method of preparation of the vaccines is significant. We may state that within our experience up to the present time fresh (not glycerolated), infected brain tissue derived from mice[3] and guinea pigs, and tissue having a high content of active virus before formolization, should be used preferably.

Duration of Induced Immunity.—As shown in Table III, twelve of thirteen guinea pigs survived an intracerebral test of 1,000 M.L.D. at least, given from 80 to 92 days after the last injection, or from 87 to 99 days after the first treatment. The only animal that succumbed to the test was one of three receiving one immunizing dose. The indication is that two injections of the vaccine are more effective than one, as will be shown again later (see also Tables III, IV, and V).

Table III presents results which refer to the duration of resistance over relatively shorter periods of time than those given in Table IV. It will be noted from the latter table that in the intervals extending from 126 to 200 days after vaccination with the formolized preparations, immunity still persisted, although somewhat reduced in degree following an intracerebral test but yet fairly solid after the subcutaneous or intranasal tests for induced resistance. When compared with the protection conferred by active virus over prolonged periods of time, from 185 to 240 days after vaccination (Table IV), the results point to the fact that while animals immunized with untreated virus withstand the intracerebral test for resistance to a higher degree, there is no significant difference between untreated and formolized virus vaccines in their respective capacities to induce protection against nasal or subcutaneous experimental infection—infection more closely simulating what occurs in nature—during the period of time studied.

Stability of Antigenic Power.—The antigenic stability of the formolized preparations is well illustrated in the records of vaccines III and XI (Table I). They retained their immunizing property after storage at 5°C. for 63 and 65 days respectively.

[3] As shown in Table I, effective vaccines were prepared from mouse brain stored in the cold in Petri plates for as long as 17 to 19 days.

In both materials the formalin was not neutralized by ammonium hydroxide until just before animal inoculation. By way of contrast, vaccine IV, neutralized just after inactivation by formalin had been completed, rapidly lost its capacity to immunize (Table II).

Relation of Dose and Dilution of Vaccines to Induced Immunity.— The results thus far tabulated consistently show a relation of the dose

TABLE III

Duration of Immunity in Guinea Pigs against Intracerebral Infection

No. of vaccine	Source of virus	M.l.u. per cc. before inactivation	Test for active virus	Immunization			Test for immunity	
				Interval between preparation and use	Route and dose	Interval between last injection and immunity test	M.L.D.	Result / No. of survivors
				days		*days*		
II	F G B	Not tested	Negative	4	2 sc, 1 cc. each, 6 d. int.	75	>10<100	1/1*
XI	F M B 5°C. 8 d.	3×10^7	"	12	2 sc, 1 cc. each, 7 d. int.	92	>1,000	4/4
XII	F M B 5°C. 17 d.	3×10^8	"	5	1 sc, 1 cc.	87	>1,000	2/3
XII	" "	3×10^8	"	5	1 sc, 2 cc.	87	>1,000	3/3
XII	" "	3×10^8	"	5	2 sc, 1 cc. each, 7 d. int.	80	>1,000	3/3

Abbreviations as in Table I.

* Only one animal of a series survived intercurrent infections during the period prior to the immunity test.

of a vaccine to the immunity induced in guinea pigs. Consequently two injections, each of 1 cc., are practical for immunization. The relation of quantity is again brought out in experiments on mice, summarized in Table V. Undiluted preparations yielded complete, whereas 10^{-1} dilution, little or no protection, and still greater dilutions none.

In vaccine IV the usual technique was modified without good results (Table II), while vaccine VIII was only partially effective; its virus content was not determined prior to formolization.

It would appear, therefore, that the method of preparation of the vaccines is significant. We may state that within our experience up to the present time fresh (not glycerolated), infected brain tissue derived from mice[3] and guinea pigs, and tissue having a high content of active virus before formolization, should be used preferably.

Duration of Induced Immunity.—As shown in Table III, twelve of thirteen guinea pigs survived an intracerebral test of 1,000 M.L.D. at least, given from 80 to 92 days after the last injection, or from 87 to 99 days after the first treatment. The only animal that succumbed to the test was one of three receiving one immunizing dose. The indication is that two injections of the vaccine are more effective than one, as will be shown again later (see also Tables III, IV, and V).

Table III presents results which refer to the duration of resistance over relatively shorter periods of time than those given in Table IV. It will be noted from the latter table that in the intervals extending from 126 to 200 days after vaccination with the formolized preparations, immunity still persisted, although somewhat reduced in degree following an intracerebral test but yet fairly solid after the subcutaneous or intranasal tests for induced resistance. When compared with the protection conferred by active virus over prolonged periods of time, from 185 to 240 days after vaccination (Table IV), the results point to the fact that while animals immunized with untreated virus withstand the intracerebral test for resistance to a higher degree, there is no significant difference between untreated and formolized virus vaccines in their respective capacities to induce protection against nasal or subcutaneous experimental infection—infection more closely simulating what occurs in nature—during the period of time studied.

Stability of Antigenic Power.—The antigenic stability of the formolized preparations is well illustrated in the records of vaccines III and XI (Table I). They retained their immunizing property after storage at 5°C. for 63 and 65 days respectively.

[3] As shown in Table I, effective vaccines were prepared from mouse brain stored in the cold in Petri plates for as long as 17 to 19 days.

TABLE V

Immunization Tests in Mice

Immunization						Intracerebral test for immunity	
Active virus			Formolized vaccine				
Route and dose	No. of m.i.u. 1st dose	No. of m.i.u. 2nd dose	Route and dose	Dilution	No. of mice surviving treatment	Interval between last injection and immunity test	M.L.D.	Result No. of survivors
						days		
2 ip, 0.2 cc. each, 7 d. int.	6×10^6	6×10^5			3/10	16	>20<50	3/3
" "	6×10^5	6×10^4			5/10	16	>20<50	5/5
" "	6×10^4	6×10^3			7/10	16	>20<50	7/7
" "	6×10^3	6×10^2			8/10	16	>20<50	8/8
" "	6×10^2	6×10^1			9/10	16	>20<50	5/9
" "	6×10^1	6×10^0			10/10	16	>20<50	2/10
" "	6×10^0	Broth			10/10	16	>20<50	0/10
	Broth	"	V. ic 0.03 cc. + 2 ip 0.2 cc. each, 7 d. int.	Undiluted	18/18	12	>20<50	0/10
			" "	1:10	9/9	12	>20<50	18/18
			" "	1:100	10/10	12	>20<50	5/9
			" "	1:1,000	10/10	12	>20<50	1/10
			" "	1:10,000	10/10	12	>20<50	0/10
			VI. ic 0.03 cc. + 2 ip 0.5 cc. each, 6 d. int.	Undiluted	12/12	10	>20<50	0/10
			VII. 2 sc 0.25 cc. each, 7 d. int.	"	10/10	22	>50<100	12/12
			VII. ic, 0.03 cc. + 2 sc 0.25 cc. each, 7 d. int.	"	10/10	22	>50<100	10/10
			" "	1:10	10/10	22	>50<100	10/10
			" "	1:100	10/10	22	>50<100	0/10
			" "	1:1,000	10/10	22	>50<100	0/10

ip = intraperitoneal; otherwise abbreviations are same as in Table I.

TABLE IV

Duration of Resistance over Longer Periods of Time than Given in Table III

Immunization			Previous immunity test				Present test for immunity		
No. of vaccine	Source of virus	Route and dose	Interval between last injection and immunity test	Route of injection	No. of survivors	Interval between last injection and present immunity test	Route of injection	M.L.D.	No. of survivors
			days			*days*			
IX	F G B	2 sc, 1 cc. each, 7 d. int.	—	—	—	200	ic	>100	1/4
XI	F M B	2 " 1 " " 7 " "	—	—	—	126	"	>100	3/5
XII	"	2 " 1 " " 7 " "	—	—	—	165	"	>100	2/5
XIV	"	2 " 1 " " 7 " "	—	—	—	137	"	>100	1/4
"	"	3 " 1 " " 7 " "	—	—	—	137	"	>100	3/4
"	"	1 or 2 sc, 1 cc. each, 7 d. int.	—	—	—	137	"	>100	3/6
"	"	1 sc, 1 cc.	31 to 38	in	7/8	137	"	>100	3/4
"	"	2 " 1 " each, 7 d. int.	38	sc	4/4	137	"	>100	3/4
"	"	2 " 1 " " 7 " "	31	"	4/4	137	"	>100	3/3
"	"	2 " 0.2 " " 5 " "	38	ic	5/5	137	"	>100	2/3
X	F G B	5 " 0.2 " " 5 " "	31	sc	4/5	137	"	>100	3/3
XII	F M B	2 " 1 " " 7 " "	16	"	5/5	150	sc	NT 4/11*	8/8
Active virus	"	2 " 1 " " 7 " "	—	—	—	196	"	" 4/11	2/2
" "	"	3 " 3×10^4 each, 7 d. int.	—	—	—	178	"	" 4/11	5/5
" "	"	3 " 3×10^5 " 7 " "	—	—	—	185 to 240	ic	>100	5/6
" "	"	3 " 3×10^4 " 7 " "	—	—	—	185 to 240	"	>100	3/4
" "	"	3 " 3×10^5 " 7 " "	—	—	—	195	in	NT 4/11	3/4
" "	"	3 " 3×10^4 " 7 " "	—	—	—	195	"	" 4/11	6/6
" "	"	3 " 3×10^5 " 7 " "	195	in	3/4	240	sc	" 1/3	3/3
		3 " 3×10^5 " 7 " "	195	"	6/6	240	"	" 1/3	6/6
			230	"	6/6	240	ic	>1,000	5/6

*In this experiment there were available two young guinea pigs born of immunized mothers, and three young of similar age and weight born of an untreated mother. Both of the former resisted a subcutaneous test, while all of the three siblings of the non-immunized mother succumbed to experimental infection. These results suggest the possibility of an immunity being conferred on the offspring of the vaccinated mother.

Abbreviations as in Table I.

inoculation of tissue cultures and mice. The experiment is summarized in Table VIII and reveals that ineffective formolized material was still inert when

TABLE VI

Comparative Antigenic Effects of Active and Formolized Virus (Specimen Pattern of an Immunity Test)

Immunization		Interval between last injection and immunity test	Intracerebral test for immunity		
Route and dose			Virus (dried) dilution	M.L.D.	Result No. of survivors
No. of m.i.u. in active virus	Formolized vaccine				
		days			
1 sc, 3×10^8		38	1:25	1,000	2/3
1 sc, 3×10^7		38	1:25	1,000	5/5
1 sc, 3×10^6		38	1:25	1,000	5/6
1 sc, 3×10^5		38	1:25	1,000	5/5
2 sc, 3×10^5 each, 7 d. int.		31	1:25	1,000	6/6
1 sc, 3×10^4		31	1:25	1,000	2/5
2 sc, 3×10^4 each, 7 d. int.		31	1:25	1,000	8/8
3 sc, 3×10^4 each, 7 d. int.		31	1:25	1,000	6/6
1 sc, 3×10^3		31	1:25	1,000	3/5
2 sc, 3×10^3 each, 7 d. int.		31	1:25	1,000	5/5
3 sc, 3×10^3 each, 7 d. int.		31	1:25	1,000	5/5
1 sc, 3×10^2		31	1:25	1,000	0/4
2 sc, 3×10^2 each, 7 d. int.		31	1:25	1,000	0/4
3 sc, 3×10^2 each, 7 d. int.		31	1:25	1,000	0/4
	XIV. 2 sc, 1 cc. each, 7 d. int.	38	1:25	1,000	5/5
Controls			1:25	1,000	0/9
"			1:250	100	0/2
"			1:2,500	10	0/2
"			1:10,000	>2<3	0/2
"			1:25,000	1	0/2
"			1:100,000	0	2/2

Abbreviations as in Table I.

known quantities of active mouse passage virus were added. Resistance to test doses was induced only when the amounts of active virus added approached the adequate dosage (5) as shown in Tables VI and VII.

Comparison of Formolized and Untreated, Active Virus

A comparison was now attempted with formolized virus on the one hand and with active virus on the other, on the respective degrees of immunity developed in mice and guinea pigs. As a corollary, the test of adding small, known amounts of virus to ineffective, formolized tissue was performed to determine whether such virus was antigenic since, as has been shown (5), these small quantities were not immunizing by themselves.

1. Tests with the Mouse.—The optimal dosage of active virus needed to protect guinea pigs uniformly against 1,000 M.L.D. given by way of the brain has already been defined in a foregoing section. Similarly to build up resistance in mice regularly against twenty to fifty intracerebral lethal doses, 600 to 6,000 m.i.u. of virus given intraperitoneally twice at 7 days' interval are required. But, as shown in Table V, such amounts of active virus, although the optimum for immunization, are dangerous to the mouse, since of ten animals receiving this treatment, two succumbed to virus infection. Even subliminal quantities produced fatal infection. On the other hand, solid resistance was obtained with safety by the use of the adequate dose of formolized vaccine, in which it was not possible to detect active virus (Table V).

2. Tests with the Guinea Pig.—Tables VI and VII summarize the results of a large number of experiments on comparison of active virus, as such, and formolized vaccines as immunizing materials. It is clear that guinea pigs were rendered resistant to as many as 1,000 intracerebral lethal doses by means of formolized vaccines in which it was impossible to demonstrate the presence of active virus. It is equally plain that to obtain the same degree of resistance by means of active virus itself, it was found necessary to inject subcutaneously 3,000 to 30,000 m.i.u. three times at 7 days' interval.

Forty guinea pigs resisted an intracerebral test of over 1,000 M.L.D. 17 to 99 days after immunization with formolized virus was begun. Thirty-seven of them withstood a repeated, similar intracerebral test 14 to 90 days following the first test, and all of six were protected against a third intracerebral dose of over 1,000 M.L.D. given 78 days after the second test (or 128 days after vaccination was begun).

3. Effect of Minute Amounts of Virus Added to Formolized Tissue.—The object of this experiment was to determine whether minute amounts of virus in the presence of large amounts of formolized tissue may be antigenic, when either by itself was non-immunizing.

Vaccine XIII was stored at 5°C. for 6 months. At the end of this time, no active virus could be demonstrated in it by inoculation of either tissue cultures or mice. To formalin-neutralized portions of the material were added known quantities of active virus and guinea pigs were injected subcutaneously with the mixtures. The presence of active virus in the mixtures was manifested by

Thus, large amounts of tissue do not activate small amounts of virus to become antigenic.

The three groups of experiments included in this section yielded results which are in agreement with each other and focus to the point that the virus, inactivated by formalin, still retains the capacity of inducing immunity. The degree of resistance produced by formolized virus is similar to that developed by untreated active virus and the protection conferred is not due to residual virus which may possibly be present in the vaccines.[4]

Recapitulation

Before discussing the experimental results, a summary of this study is indicated.

Guinea pigs can be rendered resistant to as many as 1,000 intracerebral lethal doses by the use of suitable preparations of virus which have been, in a practical sense, wholly inactivated by the addition of formalin. Suitable preparations are those made with tissue which is non-glycerolated and has a high content of virus before formolization, and in which the chemical is not neutralized until just before use in animals. These conditions have been disclosed thus far by the present experiments but the future may yet reveal other refinements in technique. The vaccines so prepared hold their antigenicity after storing for over 2 months. Not only guinea pigs but mice as well can be rendered immune by means of formolized virus.

The induced resistance is increased or diminished depending on the amount of the material given. The optimal dose for guinea pigs is two subcutaneous injections of 1 cc. each spaced about a week apart and for mice, 0.5 cc. subcutaneously or intraperitoneally at the same interval.

To obtain the same degree of protection by use of untreated active virus, it is necessary to inject guinea pigs subcutaneously with 3,000 to 30,000 mouse intracerebral units of virus three times at 7 days'

[4] A study on passive immunity (*i.e.*, antiviral bodies produced in guinea pigs by preparations containing active and inactivated virus) will be the subject of another communication. Although such bodies were found in both instances, the technical difficulties of their demonstration require an account too lengthy for this paper.

TABLE VII

Comparative Antigenic Effect of Active and Formolized Virus

Material used for immunization				
No. of weekly injections	No. of mouse infective units in active virus	Formolized vaccine	Interval between last injection and immunity test	Immunity to 1,000 or more intracerebral lethal doses
				Result: No. of survivors
1	3×10^5 to 3×10^8	—	*days* 15 to 38	21/23
2	3×10^5	—	15 to 38	12/14
3	3×10^5	—	15 to 110	13/14
1	3×10^3 to 3×10^4	—	15 to 38	8/33
2	3×10^3 to 3×10^4	—	15 to 38	15/31
3	3×10^3 to 3×10^4	—	15 to 110	64/65
1	3×10^2	—	15 to 38	0/27
2	3×10^2	—	15 to 38	0/13
3	3×10^2	—	15 to 38	2/12
2	—	1 cc. at 7 day interval	12 to 92	60/62

TABLE VIII

Formolized Vaccine to Which Known Quantities of Active Virus Were Added*

No. of weekly injections	Material used for immunization (1 cc. of vaccine or broth)	Immunity after 14 days to 1,000 or more intracerebral lethal doses
		Result: No. of survivors
2	Vaccine + broth	1/8
2	Vaccine + 30 to 150 m.i.u. in broth	1/8
2	Vaccine + 300 to 1,500 m.i.u. in broth	4/8
2	Vaccine + 3,000 to 15,000 m.i.u. in broth	7/8
2	Broth + 30 to 150 m.i.u. in broth	0/8
	Broth + 300 to 1,500 m.i.u. in broth	2/8
	Broth + 3,000 to 15,000 m.i.u. in broth	3/8

* This vaccine had been shown to be deficient in antigenic capacity (Table II, vaccine XIII).

The somewhat better results exhibited by 300 to 1,500 units of virus added to vaccine over the same amount of virus in broth may be ascribed to summation effect of two substances which, when used separately, had only little effect (Table VIII).

In a test already mentioned, part of some fresh, infected brain was immersed in glycerol and part in formalin. The formolized fresh material proved antigenic; the formolized glycerolated substance, non-immunizing. If the assumption is made that the virus content of both portions of the brain was equal before chemical treatment, it follows that formalin itself has the particular property of preserving the antigenic complex, while glycerol has not.

Again, evidence is at hand which indicates that the power of inactivated, formolized virus to immunize depends on the high virus content of the tissues with which the vaccines are made. While this factor is also noted in canine distemper (11) and Rift Valley fever (12) viruses, it is not generally applicable, since negative or inconclusive results have been reported for the antigenicity of formolized vaccinia (13) and yellow fever (14) viruses. Yet the latter two agents can be diluted as high as 10^{-7} and 10^{-8} and still be infective in susceptible animals. On the other hand, Bedson (15) protected guinea pigs with inactivated, formolized herpes virus vaccines made from tissues infective, before formolization, in limiting dilutions as low as 10^{-3} or 10^{-4}. Bedson also found that while inactivated material induced immunity, 10 M.I.D. of active herpes virus were ineffective.

It is of interest, furthermore, that when formolized vaccines are neutralized with ammonium hydroxide before storage, their antigenicity is rapidly lost. However this may be, vaccines kept in contact with formalin over long periods of time retain their immunizing capacity. Our results confirm the findings of Shahan and Giltner (16) on this point: Unneutralized vaccines were still effective after storage in the cold for at least 65 days. A similar stability has been noted with formolized Rift Valley fever (12) and dog distemper (11) viruses.[5]

[5] Bedson observed (15) that formolized herpes material is more resistant to steaming than active virus. The encephalomyelitis virus apparently differs in this respect. Forty mice in five groups of eight each were treated as follows: Mice of group I received formolized vaccine; those of group II, the same, steamed 30 minutes; group III, the same steamed 60 minutes. Mice of group IV received active virus steamed 30 minutes and group V the same steamed 60 minutes. The same virus sample entered into the preparation of both materials employed in the five groups. 10 days later, all animals were given an intracerebral test with $>10<50$ M.L.D. and only the mice of group I (formolized vaccine, not steamed) resisted the test; all mice of the other groups succumbed to fatal encephalitis.

interval, and mice with 600 to 6,000 units, twice at the same periods of time. On comparison of the duration of induced resistance brought about by formolized, inactivated virus and by untreated active virus, one finds that the protection is as solid in the one as in the other within a period of about 3 months after vaccination. During longer periods, that is, from 3 to about 7 months (the longest time thus far studied), the resistance of the animals treated with formolized vaccine is somewhat lessened against an intracerebral test as compared with that developed in animals given untreated virus but the protection against intranasal and subcutaneous test inoculation is still apparently as staunch in the first group as in the second. It should be noted, however, that the resistance conferred by active virus is not in itself absolutely solid (see Table IV, for example, which reveals that three of sixteen animals succumbed to an intracerebral, and one of four to an intranasal test, 185 to 240 days after vaccination).

Finally, small amounts of virus are not activated by large amounts of inactive, formolized tissue. This factor, taken together with other experimental findings, points to the fact that the immunity produced by the inactivated vaccines is not dependent on the presence in them of possible residual active virus.

DISCUSSION

This paper concerns itself with a study, by quantitative methods, of (*a*) the immunization of guinea pigs and mice with inactivated, formolized virus of equine encephalomyelitis and (*b*) certain phases of the mechanism underlying the development of this induced resistance.

The materials of the immunizing preparation are mouse or guinea pig passage virus treated with formalin and by rigorous tests, made as complete as is possible, found to be free from active virus. If minute amounts of active virus may have been missed by means of the methods employed for its detection, such quantities by themselves, or deliberately added to ineffective formolized tissue, fail to give rise to protection. In other words, the protection is produced by some other means than minute amounts of virus possibly residual in the vaccines.

Why such formolized preparations should be effective for immunization is not definitely known. Yet light may be thrown on the problem by reference to experiments here described and to the recorded findings with other viruses.

CONCLUSION

From a study by quantitative methods, the conclusion is reached that a resistance of high degree may be induced in guinea pigs and mice against experimental equine encephalomyelitis by means of formolized vaccines in which no active virus can be demonstrated. The induced resistance is not due to residual traces of active virus which might possibly have escaped detection in the formolized tissue preparations.

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On another point of comparison with other viruses: Laidlaw and Dunkin (11) record that vaccines prepared from distemper tissues of dogs are of little value for immunizing ferrets and similarly for ferret tissue in dogs. They believe that the lessened response to heterologous vaccines may be due to the clogging of the antibody mechanism of the recipients with multiple antigens. Shahan and Giltner (16) state that guinea pigs are usually more solidly protected against an intracerebral test by means of homologous encephalomyelitis tissue than with horse material. In our hands, both fresh mouse and guinea pig brain after formolization proved equally effective as antigens.

Since now and again one or another point is found which coincides with that existing in other formolized viruses used in preventive inoculations, and since others are widely separated, it is necessary to emphasize that the findings here reported on the encephalomyelitis virus do not necessarily apply to other viruses.

We leave now the discussion of the theoretical aspects of the problem to consider certain practical bases on which the formolized vaccine might be used in actual field immunization, especially in places where the disease is enzootic.

It has been shown that the degree of induced resistance is in proportion to the quantity of vaccine given. Yet, no experimental evidence can be adduced from the total amounts of material inoculated—in mice 0.5 to 1.0 cc., in guinea pigs, 2 cc., and in horses (16), 50 cc.—that there is a body weight/quantity of inoculum ratio required for successful immunization.

Again, experimental and epidemiological findings indicate that equine encephalomyelitis is a blood-borne infection that is transmitted by insect vectors. It is plain that a method of immunization by means of active virus is not without danger of setting up foci of infection and spread of the disease. From the work of Shahan and Giltner (7, 16) and our own, we believe that by the use of inactivated, formolized vaccines, the danger of circulation of virus or of infection during immunization is removed and that such material might render horses resistant to the natural disease for a period of time sufficiently long to be of practical value.[6]

INFECTION AND INTOXICATION

Their Influence upon Hemoglobin Production in Experimental Anemia

By F. S. ROBSCHEIT-ROBBINS, Ph.D., and G. H. WHIPPLE, M.D.

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(Received for publication, February 20, 1936)

This paper deals with infection and a variety of intoxications which do or do not modify the production of hemoglobin and red cells in experimental anemia due to blood loss. When *clinical anemias* develop in association with infection the tendency is to explain this abnormal state of the blood on the basis of blood destruction or of lack of absorption from the intestine. This is touched upon briefly in a comprehensive review of anemias by Sturgis and associates (5). We believe that the experimental data given below indicate that the essential factor is a disturbance of the *internal metabolism* which is concerned with upbuilding of the large hemoglobin molecule. There is no evidence of any significant red blood cell destruction in certain experiments (Table 3) and strong evidence that the absorption of food constituents is normal.

These anemic dogs are perfect specimens to test in this fashion, as we have long fore periods preceding the experimental or accidental infection and long after periods for adequate base line control. In such dogs the continued anemia with adequate diet intake and constant body weight gives control of digestion and absorption of the factors which go to form body protein as well as new hemoglobin and red cells. We can use the simplest possible type of "infection"—a *sterile abscess*—which gives the clinical reactions of a bacterial abscess (leucocytosis, fever and elevated nitrogenous urinary excretion) but can be terminated in 4 days with prompt healing and no danger of continuation or complications.

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but we must admit that this type of metabolic disturbance does not modify hemoglobin production in the standard anemic dog.

Methods

The routine care of these anemic dogs has been described in earlier publications (8, 10, 11) and unless otherwise stated in the clinical history the given dog is active and clinically normal. Percentage food consumption is a valuable index of the clinical condition.

The "sterile abscess" is produced by the injection of 1 cc. of turpentine subcutaneously. The reaction is prompt and the abscess may rupture or be evacuated on the 3rd to 5th day. It contains 200–300 cc. of blood tinged pus and after escape of the pus the area heals promptly in 3–4 days. The dogs are clinically slightly indisposed and may refuse a part of a day's ration but this rarely lasts more than a day or two and the clinical recovery is very prompt. Leucocytosis and fever are noted.

All abdominal operations were done under surgical ether anesthesia with the usual aseptic technique. We are indebted to Dr. C. Arthur Elden of the Department of Obstetrics and Gynecology who did the two hysterectomies.

Thyroid (Armour's desiccated) and dinitrophenol (sodium 2.4—dinitrophenoxide—Eastman Kodak) were fed in large doses which produced the recognized therapeutic effect.

EXPERIMENTAL OBSERVATIONS

The first two experiments (Tables 1 and 2) dealing with *endometritis* are much like ordinary clinical infections of moderate severity. One followed catheterization and the other developed spontaneously. As in clinical conditions there are some confusing factors like loss of blood which cannot be accurately estimated but there can be little doubt about the influence of the infection upon the building of new hemoglobin and red cells in the standard anemic dog.

Dog 24-45 (Table 1) presents an interesting story. The anemia was begun February, 1924, and was uneventful until March, 1933, when a metabolism experiment was done with several catheterizations. The dog had been permitted to return to a normal hemoglobin level (139 per cent) at this time but during April it was noted that the hemoglobin level fell slowly to 80 per cent. There was no bleeding and no blood withdrawal except for small samples. From May to August the hemoglobin level varied between 70 and 100 per cent and the dog was clinically normal and active, with a normal weight record. A little bleeding from the vagina was noted Aug. 10 which was thought to be

tion. One of the older anemic dogs developed an extensive subacute pyorrhea and at the same time a falling off in hemoglobin production was noted. The abnormal teeth were removed and the gums given proper treatment with prompt healing whereupon the production of hemoglobin returned to normal. We were inclined to attribute this to coincidence or to digestive disturbances but were lead to try some sterile abscesses in these same anemic dogs.

Our attention was finally sharply focussed on this question by a laboratory infection (*endometritis*) in one of the old anemic dogs whose history ran back 9 years. This dog retained its health and a normal food consumption but its capacity to form new hemoglobin and red cells began to fail slowly over a period of 5 months (Table 1 and clinical history Dog 24-45). Finally the dog could form no hemoglobin even on a liver diet. A vaginal discharge pointed to the uterus. Operation showed a greatly enlarged uterus with subacute inflammation of the mucosa and hysterectomy was promptly followed by a return to the normal hemoglobin production.

The crucial experiment is illustrated by Table 3 wherein an anemic dog during a fasting period in which a sterile abscess is present can produce no surplus of hemoglobin. It is well known (3) that the fasting anemic dog can produce a liberal excess of new hemoglobin (30–60 gm. per 2 weeks) which is formed obviously from body protein of the dog and in part by a *conservation* of nitrogenous material which in the normal fasting dog appears in the urea-ammonia fraction of the urine. This obviously is a function of the *internal metabolism* related to new hemoglobin production and this function is evidently disturbed and thrown out of balance by the intoxication related to the sterile abscess.

Dinitrophenol and *thyroid* medication can bring about an increased body metabolism. Various papers (2, 7) by Tainter and associates give clinical and experimental data dealing with dinitrophenol. We decided to test these substances in the anemic dog to ascertain whether this disturbance of body metabolism would in any measure modify the standard hemoglobin output under control conditions. It will be seen in Tables 6 and 7 that the fluctuations which do occur over various time intervals fall within the limits of physiological variations noted under control conditions. The doses used were large and definite clinical reactions were obtained indicating strong therapeutic effect

Mar. 28—Hemoglobin 139%, plasma volume 661 cc. Salmon bread basal ration throughout.
Apr. 3— " 110% " " 827 "
10— " 103% " " 972 "
17— " 80% " " 936 "

Apr. 17–May 29. Hemoglobin range 80–84%, plasma volume back to normal 1169 cc. (May 29).

June 5—Hemoglobin 69% No blood removal since metabolism experiment.
12— " 101%

Salmon bread diet since metabolism experiment Apr. 1.

June 12–July 24—Hemoglobin range 88–104%. Dog clinically active and normal since metabolism experiment. Food consumption 100%, no weight loss.

Aug. 10—Noticed very slight bleeding from vagina—dog believed to be in heat. See Table 1 for details of anemia and diet. There was a little vaginal bleeding as in heat during these 6 weeks.

Sept. 6—Feces examined for ova, none found. Urine: Trace albumin, moderate number of red blood cells, *pus cells*, few hyaline casts.

8—Transfusion of 15 gm. hemoglobin. Considerable bleeding 100 cc. ±, external genital tract sensitive, labia swollen. Possible abortion suspected. Ergot 15 drops twice daily.

9—Bleeding less. Ergot continued.

11—No noticeable bleeding. Ergot discontinued.

13—Tablespoonful milky bloody fluid discharged from vagina. Dog acts normal. Food consumption 100% throughout. Leucocytes ranging from 8000–10,000.

14—Transfusion 14 gm. hemoglobin.

15—Diet 300 gm. pig liver plus secondary anemia extract plus iron. Dog very lively. Food consumption 100%. Bleeding from vagina slightly increased 25–50 cc. ±.

20—Bleeding increased to 50 cc.

22—Transfusion 13 gm. hemoglobin.

25—Over Sunday bled considerably 100 cc. +. Left 23% food on *one* day only. Leucocytes 12,400. Transfusion 18 gm. hemoglobin. No weight loss during entire period—June 30, 23.4 kg., Sept. 25, 23.6 kg.

26—*Operation* (Dr. C. A. Elden). Hysterectomy, uterus greatly enlarged. One ovary removed. Anesthesia 1 hr. 45 min. Transfusion 18 gm. hemoglobin.

27—Condition fair. Temperature 39.4°. Glucose intravenously. Leucocytes *high*. Dog stands up when spoken to. Small amount food given and all consumed.

28—Leucocytes 12,500, dog more active.

TABLE 1

Infection—Endometritis

Dog 24-45. Bull, female, adult Table begins Aug. 11, 1933

Diet periods 1 wk. each	Food consumed	Weight	Plasma volume	R.B.C.	Blood hemoglobin level	Hemoglobin removed per week
Food, gm. per day	*per cent*	*kg.*	*cc.*	*mil.*	*per cent*	*gm.*
Bread 450, salmon 100, Klim 40	100	24.4	1120	5.9	93	1.4
Bread 450, salmon 100, Klim 40	89	23.4	1129	5.2	72	2.1
Bread 450, salmon 100, Klim 40	100	23.1	1320	4.9	69	2.0
Bread 450, salmon 100, Klim 40	100	23.1	1285	4.7	55	1.4
Secondary anemia extract + Fe*	100	23.7	1248		49	2.1
Secondary anemia extract + Fe*	100	23.6	1275	4.6	77	2.1
Pig liver 300, bread 350, Klim 40	100	23.6	1406	4.2	62	2.6
Pig liver 300, bread 350, Klim 40	96	23.5	1505	3.4	46	3.3
Hysterectomy—Transfusions						
Pig liver 300, secondary anemia extract + Fe†	100	22.3	1460	3.9	51	3.8
Pig liver 300, secondary anemia extract + Fe†	100	22.8	1359	4.0	56	3.6
Bread 400, salmon 150, Klim 50	100	22.4	1195	4.7	64	1.6
Bread 400, salmon 125, Klim 50	100	23.1	1200	5.0	62	42.1
Bread 450, salmon 125, Klim 50	100	23.1	1284		62	52.7
Bread 450, salmon 100, Klim 40	100	22.9	1325	4.8	58	70.8
Bread 450, salmon 100, Klim 40	100	22.8	1322		48	47.2
Bread 450, salmon 100, Klim 40	100	22.6	1370	3.7	48	1.4
Bread 450, salmon 100, Klim 40	100	22.6	1291	4.7	54	62.3
Bread 450, salmon 100, Klim 40	100	23.0	1350	4.0	48	47.1
Bread 450, salmon 100, Klim 40	100	23.0	1395	3.5	45	1.7
Bread 450, salmon 100, Klim 40	100	23.2	1304	4.2	55	36.7
Bread 450, salmon 100, Klim 40	100	23.5	1421	3.9	43	1.3

* Bread 450, salmon 100, Klim 40.

† Bread 350, Klim 40, cod liver oil 10.

Clinical History—Endometritis—Table 1.

Dog 24-45. Adult female bull. Born Nov., 1922. Experimental anemia from Sept., 1924, to Feb., 1933. Dog returned to normal blood hemoglobin level for metabolism experiment.

Mar. 6, 1933—Metabolism experiment begun. Catheterization Mar. 15, 22, 29, Apr. 1, 1933 (3).

July 17—Pig kidney feeding experiment (300 gm. daily for 2 weeks). Negative hemoglobin output (Table 2).

24—Leucocytes 15,000—previous average 9,500.

Aug. 1–12—Food consumption 100%.

11—Slight vaginal bleeding noticed. Small amount of blood on floor of cage. Dog is active and does not appear ill.

14—Leucocytes 20,400. Temperature 100.8°.

14–21—Slight vaginal bleeding continues, temperature ranging from 100.8–101.2°. Leucocytes 10,600.

23—Bloody vaginal discharge increasing. Endometritis suspected. *Operation.* Uterus removed (Dr. C. A. Elden). Transfusion before and after operation—total 42 gm. hemoglobin.

Weight loss from July 19–Aug. 23, 0.7 kg.

Food consumption Aug. 14–23 (date of operation) averages 96%.

During entire bleeding period dog did not appear acutely ill, only noticeable symptoms were leucocyte increase, temperature rise and slight bleeding.

Aug. 24—Clinical condition good. Food consumption 100%, dog began to gain weight. Note hemoglobin output—Table 2.

Sept. 5—Recovery complete and wound healed.

Surgical Specimens

Dog 27-240 (Table 2). Uterus is greatly enlarged as in Dog 24-45. It measures about 3–5 cm. in diameter and is about three times longer than normal. Both horns contain blood tinged purulent material and the mucosa is covered with purulent exudate. The lumen is greatly dilated but the walls are thickened. Studies made by Dr. L. Ackerman of the Department of Bacteriology showed pure cultures of *B. coli.*

Histological sections show evidence of a chronic inflammation with great numbers of mononuclears and plasma cells in the interglandular stroma of the mucosa. The crypts of the glands of the mucosa are greatly dilated and contain numbers of polymorphonuclears and much cell debris. Here are found colonies of bacteria. The acute change in this case predominates but there are no definite ulcers in the mucosa. The muscle coats are thickened but show no significant extension of the inflammation. No hemorrhages are seen and no phagocytes containing blood pigment.

seen in the other dog—Table 1. There were no ulcers, no phagocytes containing pigment and the bleeding from the vagina was of no significance. *Bacillus coli* was probably largely responsible but we have been unable to cause this type of endometritis by the introduction of bacteria into the normal dog's uterus.

Hemoglobin production after hysterectomy is of considerable interest.

TABLE 2

Infection—Endometritis

Dog 27-240. Bull, female, adult

Table begins June 26, 1935

Diet periods 1 wk. each	Food consumed	Weight	Plasma volume	R.B.C.	Blood hemoglobin level	Hemoglobin removed per week
Food, gm. per day	*per cent*	*kg.*	*cc.*	*mil.*	*per cent*	*gm.*
Bread 275, salmon 125, Klim 20	89	13.3	884	4.5	43	12.1
Bread 275, salmon 150, Klim 20	91	13.5	780	4.2	47	2.2
Bread 275, salmon 150, Klim 20	79	12.9	820	5.6	52	20.5
Bread 225, salmon 200, Klim 20	85	13.5	806	4.1	44	1.2
Pig kidney 300, bread 225	71	12.3	742	3.9	47	1.4
Pig kidney 300, bread 225	93	12.6	826	4.5	52	11.2
Bread 225, salmon 200, Klim 20	82	12.6	800	3.8	49	1.3
Bread 225, salmon 200, Klim 20	95	12.2	763	3.7	46	1.8
Bread 200, salmon 200, Klim 20	96	12.0	713	4.2	47	1.5
Hysterectomy—Transfusions						
Bread 250, salmon 200, Klim 20	100	11.7	699	4.2	55	2.0
Bread 300, salmon 200, Klim 20	100	12.0	713	5.4	60	40.5
Bread 300, salmon 200, Klim 20	100	12.0	683	4.9	58	27.5
Bread 300, salmon 200, Klim 20	100	12.2	718	4.5	56	12.5
Bread 350, salmon 125, Klim 20	100	12.7	755	4.8	68	22.9
Bread 350, salmon 125, Klim 20	100	12.7	779	5.5	62	46.6
Bread 350, salmon 125, Klim 20	100	13.1	780	4.8	55	34.0
Bread 350, salmon 125, Klim 20	100	13.0	800	4.6	43	23.7
Bread 375, salmon 100, Klim 20	98	13.2	800	4.6	51	14.0
Bread 375, salmon 100, Klim 20	100	13.2	822		55	26.4
	100	13.3	760	5.5	54	34.3

Clinical History—Endometritis—Table 2.

Dog 27-240. Adult female bull. Born Feb., 1928. Continuous experimental anemia Apr., 1930, to date.

Mar., 1935—Liver feeding experiment 300 gm. daily for 2 weeks results hemoglobin output of 52 gm. in contrast to last liver experiment (Dec., 1933) with 93 gm. hemoglobin output. Lextron experiment including Fe 300 mg. on May 16, 1935, resulted in 69 gm. hemoglobin output in contrast to similar test Jan. 31, 1935, when output was 78 gm. hemoglobin.

Salmon bread basal hemoglobin output—16 gm. per week, Jan., 1935
" " " " " 12 " " " Mar., 1935
" " " " " 9 " " " May, 1935

Food consumption fell off from 91% to 79% on the average.

TABLE 3

Turpentine Abscess and Fasting

Dog 24-45. Bull, female, adult

Diet periods 1 wk. each	Food consumed	Weight	Plasma volume	R.B.C.	Blood hemoglobin level	Hemoglobin removed per week
Food, gm. per day	*per cent*	*kg.*	*cc.*	*mil.*	*per cent*	*gm.*
Bread 450, salmon 50	100	21.2	1211	3.6	42	1.2
Turpentine—1 dose, fasting		19.1	1075	4.3	41	1.2
Turpentine—2 doses, fasting		17.2	980	5.6	53	13.9
Bread 450, salmon 50	100	17.6	1065	3.9	41	1.2
Bread 450, salmon 50	100	18.7	1170	3.7	35	1.0
Basal output—5 gm. Hb. per week. Total net Hb. output—0						Total = 17.3
Bread 600, salmon 50	100	23.1	1140	5.6	58	1.9
Fasting		20.6	1082	6.1	60	21.1
Fasting		18.3	901	6.4	59	18.4
Bread 400, salmon 50	100	20.0	1108	4.9	44	18.4
Bread 350, salmon 50	100	19.9	1118	5.5	49	36.6
Total net Hb. output—76 gm. Total = 94.5						

Clinical History—Sterile Abscess and Fasting—Table 3.

Dog 24-45. Adult female bull. Born Nov., 1922. Experimental anemia begun Sept., 1924, and a normal anemia history to Mar., 1927.

Mar. 22, 1927—Fasting begun, turpentine 1 cc. subcutaneously.

25—Abscess draining bloody purulent thick material. 2nd abscess, 1 cc. turpentine subcutaneously.

28—Abscess draining.

Apr. 3—3rd abscess, 1 cc. turpentine subcutaneously.

5—Returned to salmon bread diet with prompt healing and recovery.

Dog 24-46 (Table 4) gives equally convincing data to show that the sterile abscess inhibits the production of new hemoglobin during the *2nd week* of a fast and the 2 following weeks. The usual reaction due to simple fasting is noted in the 1st week when 24 gm. hemoglobin are removed. This is to be compared with the simple fasting reaction (Dog 24-45, Table 3).

If we omit the 1st postoperative week because of wound healing and take the next 10 weeks period, the total hemoglobin production is 282 gm. If we assume that the hemoglobin production due to basal salmon bread feeding is the same after as before the endometritis—namely 16 gm. per week, we can account for 160 gm. hemoglobin plus the 42 gm. hemoglobin given in transfusions = 202 gm. hemoglobin. This deducted from 282 gm. hemoglobin actually removed leaves a total of 80 gm. hemoglobin unexplained. We are inclined to attribute this 80 gm. hemoglobin to the favorable *kidney dict* given shortly *before* the operation. If this is true an important point is established—that infection under such conditions does not interfere with *absorption* and *storage* of products going to form hemoglobin but does interfere with the orderly *production* of new hemoglobin within the body (see also Table 3—fasting). This experiment would answer the question about *absorption* of hemoglobin building stones in this type of infection—the trouble is not with absorption but somewhere along the line of *protein anabolism* within the body. It must be admitted that hypothetical blood destruction might explain a part of this 80 gm. surplus as the products of the destroyed red cells would be stored in speen and liver to be utilized at a later date. However we can control this factor in the next experiment (Table 3).

Table 3 gives two important experiments to show clearly that *intoxication* (sterile abscess) disturbs the production of hemoglobin during a fasting period. Obviously the disturbance cannot influence any *absorption* and must act on some phase of *internal protein metabolism*. The contrast (Table 3) in the same dog fasting with and without the sterile abscess is striking. Dog 24-45 produces 76 gm. new hemoglobin as the result of a 2 weeks fast and at another time in the presence of these sterile abscesses can produce no hemoglobin above the basal output. It is well known (1) that there is an increased protein breakdown and increased urinary nitrogen caused by a sterile abscess but the body cannot use this nitrogenous material. Under simple fasting conditions the anemic dog can make large amounts of new hemoglobin and actually conserves for hemoglobin construction some of the nitrogenous material which otherwise in the non-anemic dog would be wasted and appear in the ammonia-urea fraction in the urine (3).

Jan. 27—Third dose of turpentine subcutaneously. Leucocytes 12,600. Temperature 102°.
28—Leucocytes 16,600. Temperature 102.2°. Normal salt solution intravenous. No bile pigment in urine.
29—No bile pigment in urine. Leucocytes 30,400.
30—Leucocytes 23,000. Temperature 101°. Given ⅓ usual ration of salmon bread diet.
31—Given ½ usual ration of salmon bread.
Feb. 1—Abscess draining. Leucocytes 11,600. Temperature 100°. Full bread ration given.
5—Healing complete. Leucocytes 9,500.

Dog 24-46. Female bull, young adult, born 1923. Continuous uneventful anemia history Sept., 1924, to Aug., 1927. Splenectomy June 16, 1925.
Mar. 29, 1927—Fasting begun.
Apr. 5—Fasting continued, 1 cc. turpentine subcutaneously.
11—Abscess draining.
12—Returned to salmon bread diet. Rapid recovery.

Dog 27-234, Table 4, gives additional evidence on the important point that the sterile abscess will stop the production of hemoglobin. This dog has a high base line production of 17 gm. hemoglobin per week on salmon bread but this animal shows no significant production of hemoglobin during the fasting period or in the 2 weeks after period. Compare Dog 24-45, Table 3, with a lower base line hemoglobin production (5 gm. per week) and yet 76 gm. hemoglobin output due to fasting alone. Dog 27-234, Table 4, showed no bile pigment in urine or blood plasma during the abscess period.

A final test of possible blood destruction due to the sterile turpentine abscess may be briefly outlined.

Dog 32-2. A *renal bile fistula* anemic for 21 months.
Feb. 1—Urine 24 hrs. 520 cc.—bile pigment content 55 mg.
2— " " " 500 "— " " " 43 "
3— " " " 530 "— " " " 42 "
Turpentine 1 cc. given subcutaneously caused the usual abscess reaction.
Feb. 4—Urine 24 hrs. 960 cc.—bile pigment content 56 mg.
5— " " " 545 "— " " " 84 "
6— " " " 675 "— " " " 92 "
Abscess incised and drained of about 120 cc. pus.
Feb. 7—Urine 24 hrs. 670 cc.—bile pigment content 70 mg.
8— " " " 600 "— " " " 50 "
Abscess healing—no urobilin at any time. Dog in excellent clinical condition.

TABLE 4

Turpentine Abscess and Fasting

Diet periods 1 wk. each	Food consumed	Weight	Plasma volume	R.B.C.	Blood hemoglobin level	Hemoglobin removed per week
Dog 27-234. Bull, male, adult						
Food, gm. per day	*per cent*	*kg.*	*cc.*	*mil.*	*per cent*	*gm.*
Bread 325, salmon 75, Klim 20	100	16.9	992	4.3	46	1.4
Turpentine—2 doses, fasting		15.0	870	4.9	45	11.0
Turpentine—1 dose, fasting		14.4	873	3.7	41	1.2
Bread 325, salmon 75, Klim 20	100	13.7	818	4.6	48	1.3
Bread 325, salmon 75, Klim 20	100	13.8	756	4.8	50	22.0
Basal output—17 gm. Hb. per week. Total net Hb. output—6 gm. Total = 35.5						
Dog 24-46. Bull, female, adult, splenectomy						
Bread 400, salmon 75	100	19.5	907	4.0	46	1.3
Fasting		17.4	902	3.9	41	24.7
Turpentine—1 dose, fasting		15.5	886	3.1	41	1.1
Bread 400, salmon 75	100	15.9	950	3.5	38	2.0
Bread 400, salmon 75	100	16.4	965	4.1	44	1.4
Basal output 3 gm. Hb. per week						

Clinical Histories—Sterile Abscess and Fasting—Table 4.

Dog 27-234. Male, adult, bull, born Jan., 1928. Continuous uneventful anemia history Oct., 1929, to date.

Jan. 16, 1936—Fasting begun, turpentine 1 cc. subcutaneously.

21—Abscess drained, second dose of turpentine 1 cc. subcutaneously.

22—Leucocytes 16,000. Temperature 101°.

23—First abscess filled again, opened, 200 cc. bloody pus withdrawn. Urine contained no bile pigment.

24—Dog is not clinically well. Two intravenous injections of 200 cc. normal salt solution. Temperature 101.7°.

25—Leucocytes 24,000. Temperature 100°. No bile pigment in urine. Abscess draining. Normal salt solution injected intravenously. Dog improved.

26—One injection of normal salt solution. No bile pigment in urine.

TABLE 7

Desiccated Thyroid—Negative Influence on Hemoglobin Production

Diet periods 1 wk. each	Experimental period	Food consumed	Weight average	Blood hemoglobin level average	Hemoglobin removed per week average
Dog 33-14. Coach, female, adult					
Food, gm. per day	*wks.*	*per cent*	*kg.*	*per cent*	*gm.*
Bread 375, salmon 75, Klim 20	17	99	11.3	52	22.2
Thyroid 15, bread 375, salmon 125, Klim 20	4	99	11.4	47	10.0
Bread 375, salmon 100, Klim 20	8	100	11.9	49	13.0
Dog 33-13. Coach, male, adult					
Bread 390, salmon 75, Klim 20	18	100	17.4	46	14.4
Thyroid 10, bread 425, salmon 75, Klim 20	4	100	16.4	45	19.4
Bread 425, salmon 75, Klim 20	11	100	17.8	48	15.9
Dog 30-114. Coach, male, adult					
Bread 450, salmon 75, Klim 25	18	100	14.2	47	11.0
Thyroid 15, bread 450, salmon 75, Klim 20	5	100	13.5	47	19.3
Bread 450, salmon 75, Klim 20	10	100	14.1	44	12.6

Clinical History—Dinitrophenol and Thyroid—Tables 6, 7 and 8.

Dog 30-114. Coach, mongrel, male. Born Sept., 1930. Continuous anemia history Mar. 1, 1932, to date. Bread basal hemoglobin output—9 gm. average in 1933.

Dec. 12, 1933—Sodium dinitrophenol feeding begun. Dose 16 mg. daily, weight 14.7 kg. Increasing doses up to 280 mg. daily added to salmon bread diet during 21 weeks. Temperature range 38.8–39.4°.

June 2—Temperature 40.1°, dog is very warm to touch but does not appear ill. Dinitrophenol feeding stopped with last dose of 280 mg. Salmon bread diet for 18 weeks thereafter.

Oct. 2, 1934—Desiccated thyroid 15 gm. daily for 5 weeks (Armour). Salmon bread diet. Thyroid to Nov. 7, 1934. Weight 14.9 kg. at start—at end of thyroid feeding period 12.7 kg. Maximum temperature 38.9°. No unfavorable symptoms.

Clinical History—Thyroid Feeding—Table 7.

Dog 33-13. Coach, male, adult, born Nov., 1932 (littermate to 33-14). Anemia history Dec. 29, 1933, to date. Uneventful.

Mar. 2, 1935—Desiccated thyroid daily feeding 15 gm. with salmon bread diet. Following giving of 11 doses dog became ill, sudden collapse. Thyroid omitted for 2 days, complete recovery.

Apr. 18—Second abscess draining.
22—Third abscess produced by 1 cc. turpentine subcutaneously.
23—Left 85% food.
24—Left 58% food. Urine examination: Trace of albumin. No casts, no red blood cells.
25—Left 36% food.
26—Abscess draining.
May 2—Healing complete. Dog clinically normal.
8 weeks later the liver feeding control (Table 4) was carried out as indicated.

TABLE 6

Turpentine Abscess and Bread Feeding

Diet periods 1 wk. each	Experimental period	Food consumed	Weight average	Blood hemoglobin level average	Hemoglobin removed per week average
Dog 30-114. Coach, male, adult					
Food, gm. per day	*wks.*	*per cent*	*kg.*	*per cent*	*gm.*
Bread 450, salmon 75, Klim 20	10	100	14.1	44	12.6
Turpentine 1 cc.—4 doses*	4	98	15.8	47	9.7
Bread 450, salmon 50, Klim 20	6	100	16.3	44	10.4
Dog 30-121. Coach, male, adult					
Bread 430, salmon 55, Klim 20	10	100	13.9	46	12.1
Turpentine 1 cc.—4 doses†	3	100	14.7	43	15.7
Bread 450, salmon 50, Klim 20	5	100	14.4	42	9.5
Dog 24-45. Bull, female, adult					
Bread 400, salmon 50, Klim 20	6	100	24.6	46	12.0
Turpentine 1 cc.—4 doses‡	3	100	24.3	50	6.0
Bread 400, salmon 50, Klim 20	4	100	24.0	42	16.0
Bread 400, salmon 50, Klim 20	8	100	24.1	46	16.5

* Bread 450, salmon 75, Klim 20.
† Bread 350, salmon 75, Klim 20, cod liver oil 10.
‡ Bread 400, salmon 50, Klim 20.

abscesses. All dogs were in good condition, food consumption complete and recovery rapid from the abscess reaction.

Tables 7 and 8 show that the accelerated metabolism due to thyroid feeding or dinitrophenol does not modify the hemoglobin output in these standard anemic dogs.

Thyroid (Table 7) was given in large doses as we learned from Dr. Wm. S. McCann that these doses were necessary to produce a definite physiological response in dogs. There was noted a definite loss of weight in all dogs (clinical histories). One dog shows a slight fall in hemoglobin output and two dogs show a slight increase but no significance is attached to moderate changes of this type as they come within the limits of unexplained physiological variations.

Sodium dinitrophenol (Table 8) was given in increasing doses and the amount noted each week is the daily average for that particular week. No modification of hemoglobin production is noted as due to this drug and we must conclude that like thyroid under these conditions it has no effect upon the hemopoietic tissues. Sufficient dinitrophenol was given to cause loss of weight and some temperature change (see clinical histories). The work of Tainter and associates (6) indicates that even with large doses of this drug no change is noted in the liver and other viscera.

DISCUSSION

There are certain difficulties in the use of the terms infection and intoxication. *Infection* obviously is appropriate to designate the disturbances noted in the two dogs with endometritis. Moreover in four dogs with this sort of endometritis pure cultures of *B. coli* were isolated from the uterus in three. The *sterile abscess* introduces a difficulty as it is not an infection in the strict sense of the word and yet this type of abscess (turpentine) gives the exact clinical picture of a bacterial abscess—fever, leucocytosis, accumulation of pus and increased nitrogenous excretion in the urine. Bacteria and turpentine both kill tissue at a local spot in the body and the reactions are obviously due to disintegration of the host protein with escape of split products (1). The term *intoxication* may be best for this condition (sterile abscess) but this term is so vague and has been used so loosely as to be in bad repute. At any rate whether we term it "infection" or intoxication the *sterile abscess* has a very clear cut clinical picture and a positive inhibiting effect upon the internal metabolism of hemoglobin construction during a fast.

We note (Table 5) that the sterile abscess depressed the hemoglobin output due to a liver diet but does not appreciably modify the basal output on the standard bread basal ration (Table 6). We may choose to believe that the dog on a liver diet is producing hemoglobin in far

Mar. 15—Thyroid 5 gm. for 8 days, increased to 7 gm. daily for 2 days.

29—Dog not acting well, thyroid reduced to 5 gm. for 3 days. Because dog appeared sick thyroid feeding was stopped. Food consumption 100% in spite of upsets. Weight at beginning of thyroid feeding 17.6 kg. at end 15.7 kg. Uneventful anemia history thereafter to date.

Clinical History—Thyroid and Dinitrophenol—Tables 7 and 8.

Dog 33-14. Coach, female, adult, born Nov., 1932. Anemia history Dec. 22, 1933, to date. Uneventful.

July 16, 1934—Began daily feeding of sodium dinitrophenol with 66 mg. increasing to 210 mg. during a period of 17 weeks with salmon bread diet. Food consumption 100%. Temperature range from 38.6–39.5°. Total weight loss 1.7 kg. Anemia history continuous.

Feb. 5, 1935—Desiccated thyroid feeding 15 gm. daily for 4 weeks with salmon bread diet. No symptoms. Food consumption 100%. Clinically normal. Weight at beginning of thyroid feeding 12.8 kg. at end 10.6 kg. Loss 2.2 kg.

TABLE 8

Sodium Dinitrophenol—Negative Effect on Hemoglobin Production

Diet periods 1 wk. each	Experimental period	Food consumed	Weight average	Blood hemoglobin level average	Hemoglobin removed per week average
Dog 30-114. Coach, male, adult					
Food, gm. per day	*wks.*	*per cent*	*kg.*	*per cent*	*gm.*
Bread 450 gm., salmon 75 gm., Klim 40 gm.	3	100	14.7	46	8.6
Dinitrophenol 71 mg.*	7	100	15.0	49	14.2
Dinitrophenol 144 mg.*	5	100	15.3	44	9.0
Dinitrophenol 235 mg.*	4	100	15.0	45	13.5
Dinitrophenol 280 mg.*	5	100	13.7	48	9.9
Bread 450 gm., salmon 75 gm., Klim 25 gm.	18	100	14.2	47	11.0
Dog 33-14. Coach, female, adult					
Dinitrophenol 75 mg.†	8	98	10.7	47	10.1
Dinitrophenol 138 mg.†	6	100	10.4	49	9.6
Dinitrophenol 210 mg.†	3	100	9.7	58	17.1
Bread 375 gm., salmon 75 gm., Klim 20 gm.	17	99	11.3	52	22.2

* Bread 450, salmon 75, Klim 40 gm.

† Bread 325, salmon 75, Klim 20 gm.

SUMMARY

Infection in human cases is often believed to be responsible for anemia. It is generally believed that lack of absorption and definite blood destruction are responsible for the anemia.

Accelerated metabolism due to thyroid or dinitrophenol does not modify hemoglobin production in these standard anemic dogs.

Endometritis lasting over many weeks will profoundly reduce the production of hemoglobin in the standard anemic dog.

A *sterile abscess* also will diminish the production of new hemoglobin in the anemic dog when liver is being fed but particularly during *fasting periods* when the usual abundant production of new hemoglobin is reduced to zero.

Impaired absorption can be excluded as a factor of any significance in certain experiments given above.

Destruction of red cells can likewise be excluded as of any significance in certain experiments given above.

These experiments point to a *disturbance of internal metabolism* related to hemoglobin building in the body as responsible for the inhibition of hemoglobin production under these conditions. We believe this same factor is often of importance in human disease.

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greater amounts than on a bread diet, that the physiological machine is working under greater stress and therefore is more susceptible to toxic agencies which disturb the internal metabolism related to hemoglobin production. The disturbance due to a sterile abscess is greatest during a fasting period and at this time it would seem that the body must be using every agency and all available material working under great stress to produce new hemoglobin. At any rate the physiological mechanism related to hemoglobin production during a fast is profoundly disturbed by the intoxication related to a sterile abscess.

Absorption of food products from the intestine is of fundamental importance and may be an important factor in the anemia of experimental or human infections. Appetite may be impaired and less food be consumed. When food consumption is 100 per cent, digestion normal and body weight maintained we believe that *absorption* is not a factor of importance as it would be necessary to postulate a selective interference with absorption of hemoglobin building material. One experiment (Table 2) gives evidence that hemoglobin building materials may be absorbed and stored during a period of infection and impaired hemoglobin production to appear later as new hemoglobin after removal of the infected uterus.

Blood destruction is an important factor to have clearly in mind in evaluating the experiments tabulated above. In the endometritis experiments (Tables 1 and 2) blood destruction may be an important factor although the evidence is against this explanation but when we deal with the sterile abscess in a fasting period this hypothetical blood destruction can be excluded as the freed hemoglobin would be promptly turned over by the anemic dog to appear in the subsequent weeks as new hemoglobin (9). These dogs with sterile abscesses show no bile pigment in blood plasma or urine. Moreover the anemic bile fistula dog given a sterile abscess shows only a slight increase in bile pigment elimination due to red cell extravasation in the area of inflammation—perhaps the equivalent of 3 gm. hemoglobin destroyed within the body. This is a trivial amount of hemoglobin when we are seeking an explanation to cover a difference of 50–70 gm. hemoglobin—the difference in the same dog (Table 3) between the fasting hemoglobin output with and without a sterile abscess.

THE EFFECT OF PROLONGED APPLICATION OF LARGE DOSES OF FOLLICULAR HORMONE ON THE UTERUS OF RABBITS*

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PLATE 47

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Up to the present, research on the follicular hormone has chiefly been concerned with the effect of a single application of hormone on the uterus. It was first of all necessary to investigate the effect of such quantities of hormone as are produced physiologically in the body. We now know that folliculin stimulates oestrus in a castrated mouse when given in a dose of 0.1 γ and that 200,000 to 300,000 mouse units, equalling 0.02 to 0.03 gm., are required to produce proliferation in man. When the application to the organism of large amounts, exceeding the quantities which are produced physiologically, is continued over prolonged periods, we are dealing with the effect of hormones as drugs. In this way we are able to demonstrate secondary effects, which may be desirable or undesirable from a therapeutical point of view. We have thus been able to demonstrate lately that folliculin application may influence the physiological monthly cycle of women by delaying menstrual onset.[1] By application of 70,000 m. u. folliculin, *i.e.*, a third of the quantity of hormone necessary for proliferation, an amenorrhoea may be produced through a delay in the menstrual onset, an effect which, therapeutically speaking, may be desired at times. This clinical observation led us to ask, what effect might be produced by prolonged application of follicular hormone. In the present paper the effects on the rabbit's uterus only are to be reported. A record of further results will be given later.

* The experiments have been carried out with the aid of the Rockefeller Foundation.

[1] Zondek, B., *Wien. klin. Woch.*, 1936, in press.

appear that an aseptic suppuration may occur after continued administration of a hormone.

4. Necrosis of the Muscular Layer.—(Fig. 3.) At the beginning only a whitish discoloration of the uterine surface is to be seen. Later there is a small solid white spot, and finally a white infiltration of the uterus, showing on cross-section a wedge-shaped area like hard cheese in appearance, with normal uterine tissue in its vicinity, towards the mesometrium. The aspect thus produced is so peculiar and striking, that, on the first occasion, we thought it the result of a faulty technique. We believed that pregnant animals had been used for the experiment, the fetus having died and necrosed during the course of treatment with folliculin which lasted for weeks. But when the same effect was produced on immature animals, it became evident that this was a reaction caused by the application of folliculin. The microscopic examination shows that the white spots represent infarct-like necrosis of the myometrium. The process starts with a pronounced hyperaemia, in which the vessels are highly distended by blood. Later, thrombosis of the vessels occurs and, as a sequel, wedge-shaped necrosis is produced in the muscular layer. The mucous membrane takes little part in this necrosis. The necrotising process usually starts in the uterine areas opposite the mesometrium, apparently preferring those places where placentation takes place following fertilisation. The white areas are disseminated throughout the uterus and are continuous with intact blue-red ones. The resulting contrast in colours produces a striking appearance.

Stress should be laid upon the fact that the four processes described above do not occur one after the other in each experiment, but may coexist in the same uterus. Thus a cystic hyperplasia of the endometrium, for instance, may be encountered at the top of one of the horns of the uterus, while 2 cm. lower down extreme hyperaemia is seen, and necrosis of the muscular wall is to be found elsewhere in the same horn.

The fact that the reactions just described can only be produced in the rabbit, not in the rat, appears important to me. Immature and sexually mature rats received large amounts of hormone (Folliculin-Menformon or Dimenformon) for weeks or months. The genital organs had grown markedly (hyperplasia); the uterine cornua showed livid discoloration, but extreme hyperaemia, thrombosis or necrosis was not to be observed. Following prolonged application of hormone the uterine mucous membrane of the rat showed marked proliferation, and the glands became more numerous; they also penetrated almost to the muscular layer, but a cystic degeneration of the glands was not observed. We thus see that different animals (rabbits, rats) react in different ways to prolonged application of folliculin. It is well known that the hormonic reactions in the various species of animals

EXPERIMENTAL

Immature rabbits, weighing 1,400 gm., and sexually mature animals, weighing 2,000 gm., received follicular hormone subcutaneously twice a week, either hormone alone (Folliculin-Menformon)[2] or the benzoic ester of the dihydrofollicular hormone (Dimenformon). The doses varied from 2,000 to 25,000 m.u. per week. The animals were laparotomised under ether anesthesia several times at intervals of a few weeks in order to ensure continuous observation of the effect of the treatment. On each occasion, a larger or smaller specimen of the uterus was extirpated, in order to confirm the progress of anatomical changes. As it might take too much space to report all the experiments in detail, we shall only give a record of each variety of experiment. The result may be anticipated. Following application of large doses of follicular hormone during the course of weeks or months, we observed the following effects on the rabbit uterus.

1. Hyperaemia.—The vessels in the myometrium may show lacunar dilatation, occupying nearly half of the muscular layer. The hyperaemia may also extend to the endometrium. Extravasal haemorrhages occur at times. Thus we observed haemorrhages between the serous and the muscular layers and, very rarely, bleeding in proliferated villi. Special attention should be called to the fact that extravasal haemorrhages very rarely occurred in spite of the application of large doses of folliculin. We shall demonstrate elsewhere how haemorrhages from the mucous membrane may be regularly produced.

2. Glandular-Cystic Hyperplasia of the Uterine Mucous Membrane.—(Fig. 1.) There is an increased number of glands; the dilatation of their lumina amounts to several times their usual size; the epithelial lining shows marked flattening. The larger glands rupture, and the leucocytes which are found in their lumina, extravasate in the tissues. The cystic hyperplasia of the uterine mucous membrane, experimentally produced in rabbits, represents a condition, analogous to that of human beings, in which persistence of the follicle associated with the resulting increase in the production of folliculin produces glandular hyperplasia. As we were able to demonstrate some time ago (1933),[3] a glandular-cystic hyperplasia of the uterine mucous membrane is also to be found in primary amenorrhoea caused by the persistence of the follicle.

3. Aseptic Suppuration in the Uterine Cavity.—(Fig. 2.) The uterus shows tremendous enlargement, being the thickness of the thumb, and is dough-like in consistence. When cut, large amounts of friable cheesy material are discharged. The muscular layer of the uterus is dilated and thinned out by the cheesy material. The endometrium is transformed to a honeycomb-like structure in the meshes of which the cheesy pulp is to be found. This consists mainly of eosinophil leucocytes and necrotic masses, in which bacteria could not be found. It would

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[2] We are indebted to the Organon (Oss) for kindly supplying large amounts of Folliculin-Menformon and Dimenformon.

[3] Zondek, B., *Acta Gynec. Skand.*, 1934, 12, 309.

in the external longitudinal part of the muscular layer. In the endometrium are hugely dilated vessels, which are hardly filled. (*b*) At another part of the uterus the longitudinal section of the muscular wall shows extreme hyperaemia with highly distended vessels, in addition thrombi with commencing organisation and incipient necrosis (white stripes). (*c*) In a third part the mucous membrane shows cystic hyperplasia.

3. Immature Rabbit, 1,450 Gm. Weight.—

Exploratory laparotomy shows the genital organs to be infantile. The animal receives 5,000 m.u. Dimenformon subcutaneously, twice a week, from Apr. 4 to June 12, 1935, 90,000 m.u. in all.

Laparotomy on June 12, 1935.—Uterine hornes are much enlarged, dark red, of the thickness of a little finger. There is at the right uterine cornu a circumscribed white infiltration. The histological examination of the latter shows incipient necrosis of the muscular wall and cystic hyperplasia of the endometrium. Further treatment: The animal receives 5,000 m.u. Dimenformon twice a week up to July 16, 1935, 135,000 m.u. in all. Then it is killed.

The macroscopic aspect appears to be quite different from that of the first laparotomy (June 12, 1935). The livid discoloration has disappeared, the uteri look pale, but are immensely enlarged, being as thick as a thumb, and are filled with a doughy fluid. When cut, white masses, looking like friable white cheese, are evacuated.

Microscopic examination: Thin muscular layer; instead of the normal endometrium, cystic spaces of various sizes, most of which are already to be seen with the naked eye. Size of the largest spaces, 2 to 3 mm. The walls of the cystic spaces are lined by pavement epithelium. These are small glands with cyst-like dilatation. Small glands with a somewhat higher epithelial lining are very scanty. A loose connective tissue is to be found in the septa, separating the spaces from each other. Aggregations of eosinophil leucocytes are scattered throughout these septa. The leucocytes are frequently migrating through the epithelial lining. The uterine cavity is highly charged with eosinophil leucocytes. In addition, desquamated epithelial cells, frequently invaded by eosinophil leucocytes, are sometimes to be found. Muscular layer: Eosinophil leucocytes are scattered all round. Pronounced hyperaemia, some haemorrhages and oedema are to be observed. Bacteria could not be found. There was aseptic suppuration in the uterine cavity.

4. Sexually Mature Rabbit, 1,800 Gm. Weight.—

The animal receives 12,500 m.u. Dimenformon twice a week from Jan. 23 to Mar. 26, 1935, 250,000 m.u. in all. On Mar. 26 the animal is killed.

The uterus shows a mottled appearance. At a distance of 1½ cm. from each other, the uterus shows white, clearly differentiated infiltrations (necrosis), alternating with blue-red areas. Anybody seeing this for the first time, will at once think of dead feti with secondary necrosis.

differ widely.[4] That hormone effects vary even among rodents, is also known. It need only be mentioned that we are regularly able to produce hematomas of the follicle in the rabbit and the mouse by way of prolan or the urine of pregnant women, but very rarely so in the rat.

Some individual protocols follow.

1. Immature Rabbit, 1,300 Gm. Weight.—

Exploratory laparotomy shows infantile ovaries and uteri. From the 4th of April to the 24th of June, 1935, the animal receives subcutaneous injections of 1,000 m.u. Folliculin-Menformon twice a week, altogether 18,000 m.u.

Laparotomy on June 24, 1935.—Uterus markedly hyperplastic, dark red, cystic at one spot, having the appearance of an early pregnancy. A specimen of the uterus is extirpated. Microscopic examination[5] shows a cystic-hyperplastic mucosa (Fig. 1). The ovaries are completely infantile. Further treatment: Twice a week 1,000 m.u. Folliculin-Menformon.

Laparotomy on July 16, 1935.—(The animal received 27,000 m.u. Folliculin-Menformon in all.) The uterus looks quite different now. The left horn contains several glossy infiltrations (necrosis) which form a lively contrast to the neighbouring dark red uterine tissue. The right horn, on the contrary, does not show any necrosis, but its redness is still darker than that of the left one. Diffuse hemorrhages are to be found between the serous and the external muscular layer.

2. Immature Rabbit, 1,400 Gm. Weight.—

Exploratory laparotomy shows the genital organs to be infantile. The animal receives 10,000 m.u. Dimenformon subcutaneously twice a week from Apr. 4 to June 6, 180,000 m.u. in all.

Laparotomy on June 24, 1935.—The genital organs actually give the impression of being monstrous. The vagina has grown enormously, is as thick as a thumb. The cornua of the uterus are dark red; above the cervix, at a distance of 1 cm. each, are two white areas which infiltrate the uterus (necrosis) (Fig. 3). Above these, two stripes looking like white coatings extend almost to the top of the uterus. The genital organs (uterus and vagina) weigh 10 gm. together, in the control animal 2.5 gm. The ovaries are infantile.

The microscopic aspect of different parts of the uterus varies considerably: (*a*) A clearly differentiated necrosis of the myometrium was found above the cervix (at the white spots). In this case the necrotising process takes place only

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[4] For details see: Zondek, B., Hormone des Ovariums und des Hypophysenvorderlappens, Vienna, Julius Springer, 1935, Chapter 37.

[5] I am indebted to Professor Sophia Getzova, chief of the Pathological Department of the Hadassah Hospital, who has kindly given her opinion on all the preparations.

(Zondek: Effect of follicular hormone on uterus)

Microscopic examination: Wedge-shaped necrosis of the muscular layer. The endometrium is spongy and oedematous. A villus of the mucous membrane has been preserved and shows extravasal mucosal bleeding, in addition to hugely distended vessels. Examination of the blue-red areas shows cystic hyperplasia of the mucous membrane and a perfect muscular layer. Weight of the uterus, 4 gm.

SUMMARY

Follicular hormone under physiological conditions produced hyperplasia of the muscular wall and proliferation of the mucous membrane of the uterus of rabbits.

The following pathological changes were brought about by prolonged application of large doses of the hormone: (*a*) hyperaemia of the myometrium and the endometrium, with occasional scanty extravasal haemorrhages; (*b*) glandular-cystic hyperplasia of the endometrium; (*c*) infarct-like necrosis of the myometrium; (*d*) aseptic suppuration in the uterine cavity.

These four processes can sometimes be found simultaneously in the same uterus, but they occur more frequently in sequence. While follicular hormone, applied in physiological doses, has a stimulating effect, prolonged application of large doses destroys the uterus.

The effects described above were only to be observed in the rabbit, not in the rat. This illustrates the fact that hormone reactions may vary in different species.

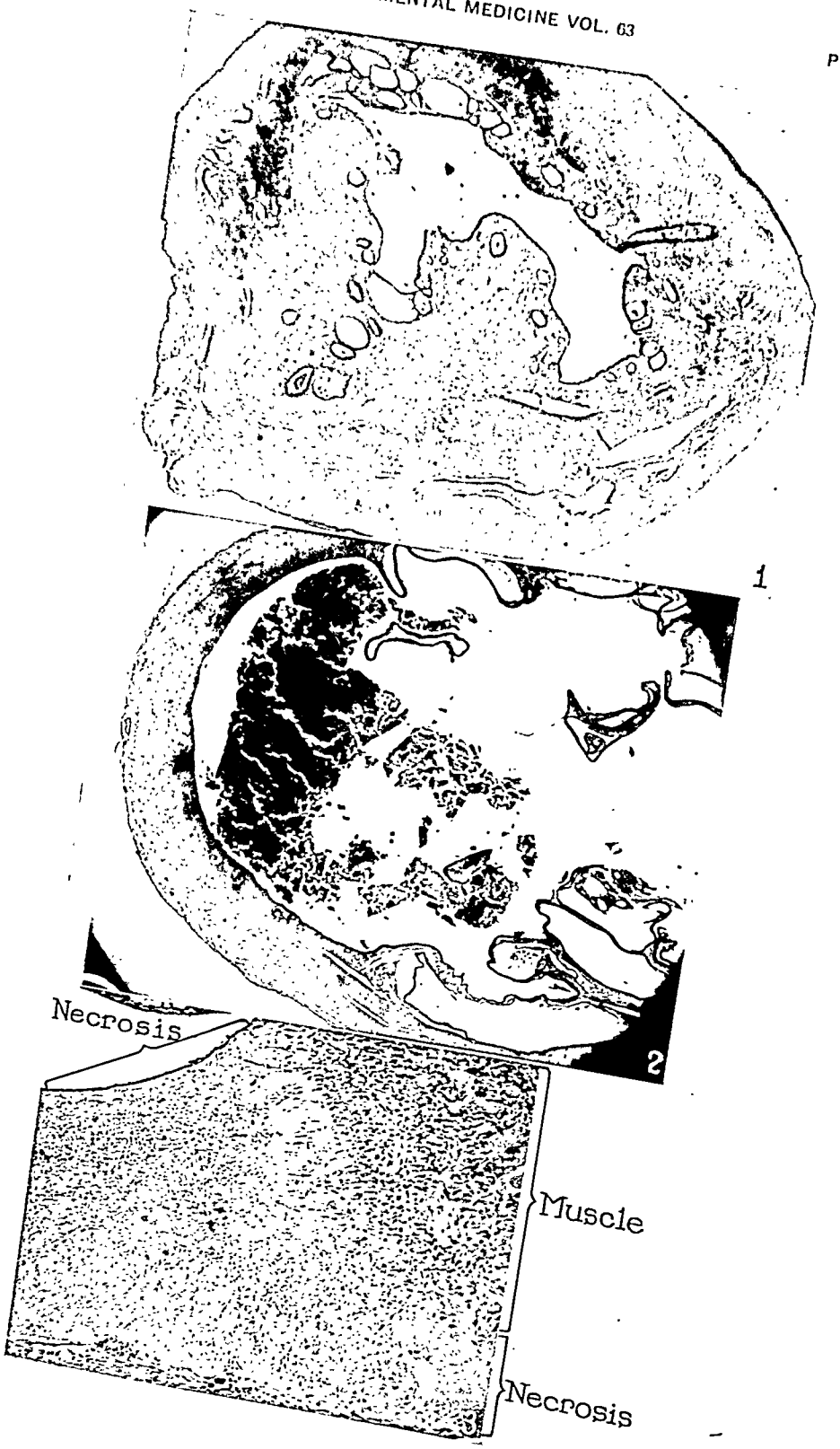
EXPLANATION OF PLATE 47

Hematoxylin-eosin stain. Zeiss microscope, ocular 10 ×, objective 8 ×.

FIG. 1. Glandular-cystic hyperplasia of the uterine mucous membrane.

FIG. 2. Suppuration in the uterine cavity.

FIG. 3. Wedge-shaped necrosis of the muscle.



(Zondek: Ef-

Microscopic examination: Wedge-shaped necrosis of the muscular layer. The endometrium is spongy and oedematous. A villus of the mucous membrane has been preserved and shows extravasal mucosal bleeding, in addition to hugely distended vessels. Examination of the blue-red areas shows cystic hyperplasia of the mucous membrane and a perfect muscular layer. Weight of the uterus, 4 gm.

SUMMARY

Follicular hormone under physiological conditions produced hyperplasia of the muscular wall and proliferation of the mucous membrane of the uterus of rabbits.

The following pathological changes were brought about by prolonged application of large doses of the hormone: (*a*) hyperaemia of the myometrium and the endometrium, with occasional scanty extravasal haemorrhages; (*b*) glandular-cystic hyperplasia of the endometrium; (*c*) infarct-like necrosis of the myometrium; (*d*) aseptic suppuration in the uterine cavity.

These four processes can sometimes be found simultaneously in the same uterus, but they occur more frequently in sequence. While follicular hormone, applied in physiological doses, has a stimulating effect, prolonged application of large doses destroys the uterus.

The effects described above were only to be observed in the rabbit, not in the rat. This illustrates the fact that hormone reactions may vary in different species.

EXPLANATION OF PLATE 47

Hematoxylin-eosin stain. Zeiss microscope, ocular 10 ×, objective 8 ×.

FIG. 1. Glandular-cystic hyperplasia of the uterine mucous membrane.

FIG. 2. Suppuration in the uterine cavity.

FIG. 3. Wedge-shaped necrosis of the muscle.

PROTHROMBIN DEFICIENCY THE CAUSE OF BLEEDING IN BILE FISTULA DOGS

By W. B. HAWKINS, M.D., AND K. M. BRINKHOUS, M.D.

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(Received for publication, February 28, 1936)

In a recent publication Hawkins and Whipple (2) stressed spontaneous bleeding as one of the important abnormalities which develops in bile fistula dogs completely deprived of bile. After 3 to 5 months of total bile deprivation there is some lengthening of the clotting time, and finally there may be spontaneous bleeding from mucous membranes or prolonged bleeding from sites of minor trauma such as a vena puncture wound. These abnormalities may be abolished within a week or two by continued feeding of dog bile (100 cc. daily). Transfusion of whole blood also will cause cessation of the bleeding, but in this case hemorrhage may soon recur. If an adequate amount of dog bile (50 to 75 cc. daily) is given from the beginning, this bleeding tendency does not develop.

It is of great interest to know what abnormality of the blood is responsible for this bleeding. Recently Warner, Brinkhous and Smith (6, 7) have developed quantitative methods for the titration of prothrombin and of antithrombin. By the use of their methods we have found a marked reduction in the amount of plasma prothrombin in our cases of bleeding. In contrast, other clotting factors–fibrinogen, calcium, blood platelets and antithrombin—have shown no significant variation from their normal levels.

Methods

The gall bladder renal type of fistula devised by Kapsinow, Engle and Harvey (5) was used. The fistula tract remains free of infection, and these dogs, properly fed, can be maintained in excellent condition for long periods of time.

The diet consisted either of salmon bread or of stock kennel rations. The salmon bread is a complete diet and its preparation has been described previously

passed. On Jan. 21, in addition to the sodium taurocholate, 1 egg yolk was added to the diet to see if a combination of these substances would influence clotting. The next day whole blood clotted slowly; in 20 minutes only a fragile clot had formed, but at the end of 40 minutes there was a solid clot which later retracted well. At this time the dog's pulse and respirations were rapid, so a whole blood transfusion (175 cc.) was given to stop the bleeding. 2 days later, on Jan. 24, the urine was free from blood. However, the delay in clotting continued. On Feb. 4, the hematuria recurred, so on the next day dog bile (50 cc.) was substituted for the bile salt and egg yolk. At this time whole blood clotted in 25 minutes. Blood oozed all that day from the vena puncture wound. The blood sample was drawn at 9:00 a.m. and at 11:30 p.m. there was still oozing of blood and a small hematoma was present in the neck. A blood transfusion (140 cc.) was given, and shortly thereafter the oozing ceased. On Feb. 9, the dog bile was increased to 100 cc. daily and no further bleeding occurred while the bile was fed. On Feb. 20, recalcified oxalate plasma (equal parts of plasma and 0.6 per cent $CaCl_2$ solution) clotted in 9 minutes. On Feb. 23, the dog bile was discontinued and 1 gm. sodium taurocholate was added to the food. 3 days later blood was present in the urine and continued to be present on succeeding days with large amounts on Mar. 2. Recalcified oxalate plasma clotted solidly in 26 minutes. On Mar. 3 the dog was found lying quietly in the cage whereas the day before it had been very active. Respirations were labored and a red cell hematocrit was only 15 per cent. A whole blood transfusion (220 cc.) was given without obvious improvement, so 2 hours later more blood (270 cc.) was given. Respirations still remained labored and in the evening the dog was found dead. In the anterior mediastinum there was a large mass composed of fat infiltrated with blood, much of which was still fluid, and in the pleural cavities there were 750 cc. of fluid blood preventing expansion of the lungs. When the heart was opened well formed clots were found within the chambers. No bleeding points could be demonstrated in the urinary tract to explain the hematuria.

It is seen from this history that ox bile and sodium taurocholate in the amounts given did not prevent the abnormality in blood clotting from developing. This bleeding tendency was controlled successfully by the feeding of whole dog bile. Whole blood transfusions were also of value, but when given alone only temporary improvement resulted. The beneficial effect of transfusion was strikingly demonstrated at the time of autopsy. The blood in the mediastinal fat and pleural cavities was unclotted and after sitting 24 hours in a flask only a flimsy clot formed. This is in contrast to the solid clots formed within the heart.

This dog was carefully studied with regard to the clotting factors from January 24 to February 5. During this period there was marked lengthening of the coagulation time (20–25 minutes), with episodes of

(8). It contains wheat flour, potato starch, bran, canned salmon, sugar, canned tomatoes, yeast, cod liver oil and a salt mixture. The bread contains 10 per cent protein, 6.5 per cent fat and 83.4 per cent carbohydrate. It is a very suitable diet for fistula dogs, since it is so rich in carbohydrates. Dogs as a rule are fed 300 to 400 gm. of this salmon bread plus 75 gm. of salmon and 30 gm. of whole milk powder (Klim). The kennel ration is composed of mixed hospital scraps and it contains much bread, potato, vegetables, a little meat and variable amounts of butter.

The prothrombin of plasma was titrated according to the method of Warner, Brinkhous and Smith (7). It is expressed in per cent of prothrombin of a normal control dog plasma. Fibrinogen was determined by the method of Jones and Smith (3). For the determination of the antithrombic activity of plasma, a known thrombin was incubated with oxalated plasma which had been dialyzed several hours against 0.9 per cent NaCl solution. The thrombin remaining was then titrated. The amount of thrombin destroyed is a measure of the antithrombin of the plasma. This method of determining antithrombin has been outlined briefly by Smith, Warner and Brinkhous (6) and will be published in detail soon. The method of Clark and Collip (1) was used for serum calcium determinations.

EXPERIMENTAL OBSERVATIONS

Most of the dogs studied had been followed in the bile pigment or anemia colonies at the University of Rochester for considerable periods of time. For purposes of brevity and clarity only the data pertinent to this problem will be given. It is well to add that the dogs were in excellent physical condition during the period of these studies. Seven dogs in all were studied. Three of these experiments, representative of the various states found, are presented in detail.

Dog. 33-90. This dog, weighing 18.6 kilos, was fed 600 gm. of kennel diet daily. It was operated upon Mar. 13, 1934, and a gall bladder renal fistula was made. The dog was continued on the kennel diet for nearly 7 months, except for a 2 week period in June when 200 gm. of pig liver and 200 gm. of salmon bread were substituted. Then, beginning Oct. 3, the kennel diet was replaced by the salmon bread mixture. This diet was fed, with the addition of pig liver (300 gm. daily) from Dec. 12 to Jan. 15, until the death of the animal.

For the first 19 weeks postoperatively, the dog was given no bile. However, during part of this time (Apr. 10 to May 20), 1 to 3 gm. of sodium taurocholate were added daily to the food. Starting July 24, ox bile (50 cc.) was given by stomach tube 6 days a week. This was continued for almost 6 months until Jan. 15, 1935. On Jan. 9, it was found that whole venous blood, placed in small clean test tubes clotted solidly in 13 to 15 minutes. 6 days later, the ox bile was replaced by 1 gm. of sodium taurocholate. Then, on Jan. 18, bleeding occurred for the first time. Blood was present in the urine, and during the next 5 days much blood was

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Nearly 22 months after the fistula was established, bleeding from the jugular vein was done repeatedly and thus in the next 26 days the hemoglobin percentage was brought down to 50 per cent (13.8 gm. hemoglobin = 100 per cent). During the first 9 days of the anemia period the bile feeding was continued, but on Dec. 1, 1934, it was replaced with 1 gm. sodium taurocholate daily. On Dec. 12, there was oozing of blood from a vena puncture wound. Blood transfusion of 40 cc. was given and the dog bile (50 cc.) was restored to the diet. Again, 4 days later, bleeding was noted following a vena puncture. Another transfusion (50 cc.) was given and the bile was increased to 70 cc. daily. The spontaneous bleeding did not recur, so on Jan. 2, 1935, the bile feeding was reduced to 50 cc. Beginning Jan. 9 the sodium taurocholate was increased from 1 gm. to 2 gm. daily. The anemia was maintained by carefully spaced bleedings. On this regimen, no further spontaneous bleeding occurred. The animal was followed in this experiment until Feb. 4, 1935.

This dog, a bile renal fistula of 2 years' duration, showed no bleeding tendency as long as the diet included adequate amounts of bile. When the dog was made anemic and no bile was given, prolonged bleeding from vena puncture wounds occurred. Two small blood transfusions and restoration of bile feeding checked the bleeding tendency during the final 7 weeks of the experiment. Unfortunately, analysis of the clotting factors was not made until after the period of spontaneous bleeding. But even after disappearance of the clinical tendency to bleed, the prothrombin level was still low, 16 per cent of normal. No doubt it was much lower than this at the time of bleeding. Other clotting factors were found to be normal at the time of our analysis (February 1 to February 4). Titration of antithrombin showed that both this plasma and a normal control plasma gave the same rates of thrombin destruction. Plasma fibrinogen was 369 mg. per cent. At this time whole blood clotted solidly in 9 minutes.

Dog 31-359. A gall bladder renal fistula and splenectomy were done Sept. 27, 1932. Beginning immediately, dog bile (50 cc.) was given 6 days a week by stomach tube. This was continued for nearly 15 months, with a supplement of ox bile (50 cc.) during the last 37 days of this period. Then, beginning Dec. 21, 1933, only the ox bile (50 cc.) was given. This was continued until the death of the animal 14 months later. The dog was fed the kennel diet, except for a period early in its course (Oct. 12, 1932, to Apr. 30, 1933), when salmon bread was substituted. The dog was killed with ether anesthesia on Feb. 25, 1935, since marked osteoporosis with multiple fractures had developed because of long continued partial bile deprivation. No spontaneous bleeding had occurred, however.

spontaneous bleeding. Although the coagulation time was greatly lengthened, a fairly firm clot finally formed. That there was no fibrinogen deficiency was further shown by a plasma fibrin value of 322 mg. per 100 cc. In titration of antithrombin, the same rate of destruction of thrombin resulted both with this plasma and with a normal control plasma. Titration of prothrombin, however, revealed a very marked hypoprothrombinemia. During this period the prothrombin level was always below 5 per cent of the normal control. Practically identical prothrombin values were obtained on study of the globulin fraction—a fraction which normally contains nearly all the prothrombin but almost no antithrombin. In this way we showed that neither antithrombin nor heparin interfered with the ordinary prothrombin titration.

As a supplement to this experiment we added prothrombin to this abnormal whole blood.

The prothrombin was prepared by fractional precipitation of plasma globulin with $(NH_4)_2SO_4$. It was dissolved in isotonic saline and dialyzed against saline for several hours to remove the excess of salts. The preparation was calcium free and failed to clot oxalated plasma. Titration showed it to contain somewhat more than twice as much prothrombin as normal whole blood.

Tube 1.—3 cc. whole blood—fragile clot in 25 minutes.

Tube 2.—2 cc. whole blood + 1 cc. saline—fragile clot in 22 minutes.

Tube 3.—2 cc. whole blood + 1 cc. prothrombin—began to clot in 4 minutes 45 seconds; clot very solid in 5 minutes 45 seconds. In tube 3, then, the prothrombin concentration was nearly equal to that of normal whole blood.

The prompt restoration to a normal clotting time strongly suggests that prothrombin alone is responsible for the abnormal state of clotting.

Studies on other dogs of this type with a marked hypoprothrombinemia showed that both serum calcium and blood platelets are within the limits of normal.

Dog 32-74. Splenectomy was performed on Nov. 29, 1932, and a gall bladder renal fistula was made on Feb. 2, 1933. Beginning 11 days later, ox bile (50 cc.) was given by stomach tube 6 days a week and this was continued for the next 21½ months. During the earlier part of this period (Mar. 16 to Dec. 18) the ox bile was supplemented with dog bile (50 cc.). The kennel diet, which was fed during the greater part of this time, was changed to the salmon bread diet on Aug. 2, 1934.

Snell and Hoerner (4) have referred to a hemorrhagic tendency in patients with a biliary fistula, but without jaundice. This picture is analogous to that produced in these experiments. It seems likely that a hypoprothrombinemia associated with the bile deprivation is also responsible for the bleeding in these cases.

SUMMARY

The bleeding tendency that develops in bile fistula dogs, completely or partially deprived of bile, is due to a prothrombin deficiency of the blood plasma. However, a relatively low prothrombin level may exist in these animals without the occurrence of spontaneous hemorrhage. The prothrombin level may remain within the limits of normal if sufficient bile has been fed. In these cases no disturbance in blood clotting occurs.

We are indebted to Dr. E. D. Warner, of the State University of Iowa, for the potent prothrombin preparation used in this study.

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The above dog was fed either dog bile or ox bile or both during the entire time, nearly $2\frac{1}{2}$ years, it had a bile renal fistula. No clotting abnormality was noted at any time. Thus, in this respect, the bile feeding was quite adequate to protect the animal. A short time before the dog was killed a normal prothrombin content of the plasma was found. Thus, on Jan. 25, 1935, it was 92 per cent of normal. We conclude that when bile is fed in suitable amounts, a normal prothrombin level may exist in the presence of a biliary fistula.

DISCUSSION

The abnormal blood clotting in bile fistula dogs is related without doubt to the bile deprivation. If an adequate amount of bile (50 to 75 cc.) is fed daily the blood continues to clot normally. But if the bile feeding is inadequate, the dogs gradually develop a bleeding tendency. This bleeding can then be controlled successfully by feeding whole dog bile (50 to 100 cc.). Ox bile appears to be less effective than dog bile in protecting these animals. Studies are now in progress to determine what factor in bile is responsible for its protective action.

In cases showing a bleeding tendency, analysis of the clotting factors reveals that the fibrinogen, calcium and platelets are normal, and that no excessive antithrombic activity is present. There is, however, a very marked diminution in the amount of prothrombin. This deficiency undoubtedly is responsible for the delay in clotting and hemorrhage. A hemorrhagic tendency does not develop, however, unless the prothrombin level is very low. This indicates that a wide margin of safety exists between the normal level of prothrombin and the critical level at which a disturbance in clotting results. This is in accord with the analysis of the prothrombin level of normal plasma recently made by Warner, Brinkhous and Smith. They have shown that plasma contains a great excess of prothrombin and that only a small fraction of it is required to give a normal clot. This would explain the absence of spontaneous bleeding in cases showing a relatively low prothrombin level. Likewise, it would explain the temporary benefit derived from blood transfusions. Here there is probably a transitory elevation of the prothrombin to a level at which blood will clot promptly.

In connection with this study, it is of interest to note that Judd,

STUDIES WITH HUMAN INFLUENZA VIRUS CULTIVATED IN ARTIFICIAL MEDIUM

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(Received for publication, January 14, 1936)

A previous report (1) has recorded the successful cultivation of a strain of human influenza virus (P.R.8) (2) in an artificial medium composed of Tyrode's solution and minced chick embryo. The virus cultivated under these conditions was found to retain its capacity to infect mice and ferrets, and it was maintained at about the same concentraton as in the lungs of infected mice from which it had been derived.

Since the preliminary report the P.R.8 strain of human influenza virus has been continuously transferred at 2 day intervals until it is now in the 70th generation. In addition, under similar conditions, a 2nd strain of human influenza virus (Philadelphia) (3) has been cultivated through 45 successive transfers in the artificial medium. Still more recently the virus of swine influenza (4), obtained from the lungs of infected mice,[1] has also been transferred to tissue culture. Burnet (5) and Smith (6) have reported the successful cultivation of strains of human influenza virus upon the chorioallantoic membranes of developing eggs. The latter has also confirmed our results in cultivating the virus in a fluid tissue medium.

Certain observations have been made regarding the conditions affecting multiplication and the time of survival of the virus. Furthermore, studies have been made of the immunological properties of culture virus as compared with the same strain of virus maintained solely by passage through susceptible animals. The present paper comprises primarily the results of these latter investigations.

Methods and Materials

Culture Medium.—The medium employed is that devised by Li and Rivers (7) based upon the earlier procedure of Maitland and Maitland (8). Chick em-

[1] Through the courtesy of Dr. Shope.

time lesions were not observed in mice inoculated with the material, but when these cultures were transplanted to fresh medium in tightly stoppered test tubes and transferred at 4 day intervals, the virus regained its full potency and was satisfactorily maintained by this procedure. However, if vaseline seals are placed over the culture medium so as to approach anaerobic conditions, the virus does not multiply, and the culture fluid after 48 hours is not infectious for mice.

The effect of variations in the amount of tissue used in the culture medium has not been fully investigated. As previously recognized, this factor plays a definite rôle in the problem of virus cultivation. A very small number of living cells apparently does not support multiplication of the virus, while too great a quantity of tissue is also detrimental. In the present study embryos of 12 to 13 days have appeared to be the most satisfactory, possibly because the embryo contains at this time a greater amount of serum which may serve to protect the virus.

Maintenance of Virulence of Human Influenza Virus in Tissue Culture

The virulence of the culture virus, or the retention of its capacity to produce pulmonary lesions in susceptible mice, has been measured at frequent intervals. In most instances, mice receiving the undiluted culture fluid succumb in 6 days or less and exhibit extensive involvement of the lungs. Titrations of the virus concentration of the standard P.R.8 cultures made at different times are presented in Table I.

It can be seen that a comparatively constant titer of between 1:1000 and 1:10,000 has been maintained.

Ferrets, as well as mice, have been successfully inoculated with virus of the 6th, 37th, and 54th transfers of the P.R.8 strain. In each case the ferret responded with fever, and in those instances in which autopsies were done involvement of the lungs of the ferret was observed. Furthermore, the serum of ferrets recovering from infection with the culture virus was found to contain a high concentration of antibodies effective against the regular mouse passage virus and the animals were found to be actively immune to reinfection when tested with ferret passage virus.

bryos after 10 to 14 days' incubation are removed aseptically from the egg, the eyes are taken out, and the remainder of the embryo is finely minced in 2 to 4 cc. of Tyrode's solution, depending upon the size of the embryo. After the preliminary period, embryos of 12 to 13 days were uniformly used. To 4.5 cc. of Tyrode's solution in a Rivers flask or in a 50 cc. Erlenmeyer flask, are added 4 drops (approximately 0.25 cc.) of the suspension of embryonic tissue. To the medium is then added 0.5 cc. of the virus-containing material. The flasks are stoppered with firm plugs of cotton bound in gauze. After 2 days' incubation at 37°C., transfers of 0.5 cc. of the culture are made to flasks containing 4.5 cc. of freshly prepared medium. For routine purposes the transfers have subsequently been made at 2 day intervals. In this manner, the P.R.8 strain has been actively maintained in culture for over 4 months and the Philadelphia strain for a somewhat shorter period. A control culture not containing virus has also been transferred as routine.

Method of Titration.—The presence of active virus in the culture fluid has been demonstrated by the instillation of the material into the nostrils of white mice lightly anesthetized with ether. The active agent induces in these animals gross pulmonary consolidation which varies in extent with the concentration of the virus. Mice receiving 0.05 cc. of culture of the usual titer die or are moribund in 4 to 6 days with complete involvement of the lungs. All mice surviving on the 6th day are sacrificed and their lungs examined for gross lesions. With cultures of lower titer only slight lesions may be produced in this time. For purposes of titration the limit of infectiousness has been taken to be the highest dilution of culture which produces visible areas of involvement in the lungs of inoculated mice. It is realized, however, that this arbitrary limit may not be entirely accurate, for it has been possible to demonstrate, by passage to normal mice, the presence of virus in the lungs of mice in which at the end of 6 days visible lesions were not observed. Nevertheless, for practical purposes, the end-point as measured by the above method has been generally employed.

Multiplication and Survival of Virus under Different Cultural Conditions

Under the standard conditions adopted, the greatest concentration of virus in the artificial medium is usually attained in 36 to 48 hours. In 72 hours a decrease in the amount of virus has begun, and after 5 to 6 days it is difficult to demonstrate active virus in the culture fluid. Nevertheless, in cultures removed from the incubator after 48 hours' incubation and placed in the refrigerator at +4°C., the virus has been found to retain practically full infectiousness for as long as 23 days. Furthermore, cultures made with the same medium in ordinary test tubes plugged with a tight rubber stopper and incubated at 37°C. have been found to contain active virus as long as 18 days. At this

time lesions were not observed in mice inoculated with the material, but when these cultures were transplanted to fresh medium in tightly stoppered test tubes and transferred at 4 day intervals, the virus regained its full potency and was satisfactorily maintained by this procedure. However, if vaseline seals are placed over the culture medium so as to approach anaerobic conditions, the virus does not multiply, and the culture fluid after 48 hours is not infectious for mice.

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It can be seen that a comparatively constant titer of between 1:1000 and 1:10,000 has been maintained.

Ferrets, as well as mice, have been successfully inoculated with virus of the 6th, 37th, and 54th transfers of the P.R.8 strain. In each case the ferret responded with fever, and in those instances in which autopsies were done involvement of the lungs of the ferret was observed. Furthermore, the serum of ferrets recovering from infection with the culture virus was found to contain a high concentration of antibodies effective against the regular mouse passage virus and the animals were found to be actively immune to reinfection when tested with ferret passage virus.

bryos after 10 to 14 days' incubation are removed aseptically from the egg, the eyes are taken out, and the remainder of the embryo is finely minced in 2 to 4 cc. of Tyrode's solution, depending upon the size of the embryo. After the preliminary period, embryos of 12 to 13 days were uniformly used. To 4.5 cc. of Tyrode's solution in a Rivers flask or in a 50 cc. Erlenmeyer flask, are added 4 drops (approximately 0.25 cc.) of the suspension of embryonic tissue. To the medium is then added 0.5 cc. of the virus-containing material. The flasks are stoppered with firm plugs of cotton bound in gauze. After 2 days' incubation at 37°C., transfers of 0.5 cc. of the culture are made to flasks containing 4.5 cc. of freshly prepared medium. For routine purposes the transfers have subsequently been made at 2 day intervals. In this manner, the P.R.8 strain has been actively maintained in culture for over 4 months and the Philadelphia strain for a somewhat shorter period. A control culture not containing virus has also been transferred as routine.

Method of Titration.—The presence of active virus in the culture fluid has been demonstrated by the instillation of the material into the nostrils of white mice lightly anesthetized with ether. The active agent induces in these animals gross pulmonary consolidation which varies in extent with the concentration of the virus. Mice receiving 0.05 cc. of culture of the usual titer die or are moribund in 4 to 6 days with complete involvement of the lungs. All mice surviving on the 6th day are sacrificed and their lungs examined for gross lesions. With cultures of lower titer only slight lesions may be produced in this time. For purposes of titration the limit of infectiousness has been taken to be the highest dilution of culture which produces visible areas of involvement in the lungs of inoculated mice. It is realized, however, that this arbitrary limit may not be entirely accurate, for it has been possible to demonstrate, by passage to normal mice, the presence of virus in the lungs of mice in which at the end of 6 days visible lesions were not observed. Nevertheless, for practical purposes, the end-point as measured by the above method has been generally employed.

Multiplication and Survival of Virus under Different Cultural Conditions

Under the standard conditions adopted, the greatest concentration of virus in the artificial medium is usually attained in 36 to 48 hours. In 72 hours a decrease in the amount of virus has begun, and after 5 to 6 days it is difficult to demonstrate active virus in the culture fluid. Nevertheless, in cultures removed from the incubator after 48 hours' incubation and placed in the refrigerator at +4°C., the virus has been found to retain practically full infectiousness for as long as 23 days. Furthermore, cultures made with the same medium in ordinary test tubes plugged with a tight rubber stopper and incubated at 37°C. have been found to contain active virus as long as 18 days. At this

Experiment 1.—To each of 15 mice was administered subcutaneously 0.3 cc. of P.R.8 virus of the 3rd culture transfer. 9 days and 21 days later, 0.3 cc. portions of the fluid of the 7th and 13th generations respectively were given intraperitoneally. 14 mice of the same stock were kept as controls, receiving no inoculations. 8 days after the last injection both vaccinated and control mice were given 0.03 cc. of a 10 per cent suspension of P.R.8 mouse passage virus intranasally. By the 10th day 11 of the 14 control mice had died with extensive pulmonary involvement; the 3 surviving control mice were killed and marked pulmonary lesions were exhibited. The vaccinated mice had appeared perfectly well, and in the 5 killed on the 10th day no pulmonary lesions were observed.

Experiment 2.—Three groups of 10 mice each were used. One group received, at 10 day intervals, 0.2 cc., 0.3 cc., 0.3 cc., of the 40th, 45th and 49th generations, respectively, of the P.R.8 culture virus subcutaneously; those of the second group received equal amounts of the same active virus cultures intraperitoneally at the same intervals. Animals of the third, or control, group, were given subcutaneously 0.2 cc. of culture medium which contained no virus, and at 10 day intervals thereafter two doses of 0.3 cc. each of similar material were given intraperitoneally. 10 days after the last vaccination all mice were given 0.03 cc. of 10 per cent suspension of P.R.8 mouse passage virus intranasally. By the 10th day after infection, 9 of the 10 control animals had died with typical pulmonary involvement; 2 of the mice which were vaccinated subcutaneously had died, although the others of this group appeared perfectly well; all of the mice which had been vaccinated by the intraperitoneal route remained well throughout the period of observation.

The results cited, and other experiments of a similar nature, have shown conclusively that mice vaccinated subcutaneously or intraperitoneally with human influenza virus transferred through many generations in tissue culture develop a staunch immunity against intranasal infection with large doses of the same strain of virus maintained entirely by serial passages in mice.

Experiments relating to the efficacy of the culture virus in the immunization of ferrets have shown that, following subcutaneous inoculation of the artificially cultivated agent, the animals develop an active resistance which distinctly modifies the disease produced by intranasal instillation of regular ferret passage virus.

Studies in progress regarding the vaccination of human individuals (11) have revealed that, following the subcutaneous inoculation of virus culture fluid, the human subject responds with a development of antibodies capable of neutralizing the virus as demonstrated by mouse protection tests.

Immunization Experiments with Culture Virus

It has been reported previously that mice inoculated subcutaneously or intraperitoneally with human influenza virus (9, 10), although showing no evidence of experimental disease, develop an immunity

TABLE I

Titrations of P.R. 8 Culture Virus at Intervals during the Course of Cultivation

Transfer	Mouse No.	Dilution of virus					
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
6th	1	*++++	±	+	0	0	
	2	*++++	++	+	±	0	
	3	++++	+++	+	±	0	
10th	1	++++	++	0	0	0	
	2	+++	++++	±	0	0	
	3	+++	++	+	0	0	
19th	1	++	+++	0			
	2	+++	±	±			
	3	+++	+	+			
31st	1	*++++	+++	+±	±		
	2	++++	+++	+	±		
39th	1	*++++	+++	++±	0		
	2	+++±	+++	++	++		
42nd	1	*++++	++	++	+		
	2	+++	++	+	±		
55th	1	*++++	*++++	+++	+	0	0
	2	*++++	++++	+++	++	0	0

0 = no gross pulmonary involvement.
± to ++++ = progressive degrees of pulmonary involvement.
* = mouse died.

which is effective against the virus inoculated intranasally. It was of interest, therefore, to determine whether the virus propagating in tissue culture outside the animal body was still capable of exerting this effect.

after culture and was then returned to artificial cultivation for 4 generations. The results are presented in Table II.

It is readily observed that normal ferret and normal rabbit serum exerted a stronger neutralizing effect against the older culture virus than against the more recent culture. Furthermore, the serum of swine convalescent from swine influenza virus infection, and of two human beings, H.F. (acute) and T.F. completely protected against the older culture virus.

When the virus from the 15th generation in artificial medium was passed through mice for 3 transfers and comparative tests were again

TABLE III

Capacity of Various Sera to Neutralize Culture Virus (P.R. 8) before and after Passage through Mice

Serum	Culture virus (21st transfer)			Culture virus (15th transfer) after 3 serial mouse passages		
	Mouse No.			Mouse No.		
	1	2	3	1	2	3
H. F. (acute)	0	0	0	+++	++	++
H. F. (convalescent)	0	0	0	0	0	0
S. S. "	0	0	0	±	0	0
T. F. (normal)	0	+	±	+++	++	++
Normal rabbit 1-29	++	++	++	+++	++++	++++
Immune (P.R.8) rabbit 1-29	0	0	0	0	0	0
Normal swine	++++	++	++	*++++	+++	*++++
Swine, immune to swine influenza	±	0	+	++	++	+++

done, it was found that after animal passages the virus had regained its original characteristics and that neutralization of the virus by these sera was no longer effected (Table III).

Recently, the conditions of cultivation have been somewhat more constant than earlier in the study, and a comparative test was made between the same strain of cultivated virus in its 54th generation and a new culture which was only 5 transfers (10 days) removed from animal passage. In this instance, the older culture virus which had exhibited little fluctuation in its potency for a considerable time, was less easily neutralized than previously and was no more susceptible to the action of serum than the younger culture (Table IV).

Studies of the Immunological Characteristics of Human Influenza Virus Grown in Artificial Medium

The evidence heretofore obtained, through titration in white mice, of the concentration of active agent in culture fluid has indicated that the virus multiplying in tissue culture reaches a concentration and maintains a virulence closely resembling that of the virus in the lungs of infected mice. However, when the capacity of certain sera to neutralize the culture virus was compared with the capacity of these sera to neutralize the same strain of mouse passage virus, distinct

TABLE II

Comparison of Neutralizing Capacity of the Same Sera Tested against Culture Virus of 4th and 15th Transfers

Serum	Culture virus (P.R. 8)					
	15th transfer (Original culture)			4th transfer (New culture)		
	Mouse No.			Mouse No.		
	1	2	3	1	2	3
Normal ferret 1-05	+	++	++	++++	+++	+++
Immune " 1-14	0	0	0	0	0	0
Normal rabbit 1-33	++++	+	+	++++	+++	++++
Immune " 1-29	0	0	0	0	0	0
" swine 14-44	0	0	0	++	++	+
Human—H.F. (acute influenza)	0	0	0	+	0	0
" H.F. (convalescent influenza)	0	0	0	0	0	0
Human—T.F. (normal)	0	0	0	—	—	—

differences were noted. In general, the neutralizing effect of the serum was enhanced when tested against culture virus, so that sera which exhibited little or no protective capacity against mouse passage strain might protect mice completely against the cultivated virus.

In the earlier phases of this study it appeared that the ease with which the virus grown in artificial medium could be neutralized by a given serum was somewhat related to the length of time it had been removed from animal passages. Tests were made simultaneously with the same sera against one culture in its 15th transfer and a culture of the same strain which had been transferred through mice

which it was derived. The culture virus is still virulent for mice and ferrets; it is capable of inducing an active state of immunity in animals vaccinated subcutaneously or intraperitoneally; it elicits specific neutralizing antibodies in the serum of infected or vaccinated animals.

The virus has been successfully cultivated to date only in the presence of oxygen; when conditions of reduced oxygenation are imposed by the use of vaseline seal, with or without the addition of cystein, multiplication of the virus is not supported. On the other hand, it has been possible to cultivate the virus in the medium of Li and Rivers in ordinary test tubes. This affords a greatly simplified procedure, since the interval between transfers may be prolonged.

The results of neutralization tests with various sera and the culture virus are presented and discussed.

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These results suggest that the variations observed have been due entirely to alterations of a quantitative, not of a qualitative nature in the virus. Certainly no change in the immunological characteristics has been observed which could be interpreted as due to an alteration in the antigenic constitution of the virus. On the other hand, since the titer of the culture virus, as measured by the customary method in susceptible mice, has remained at a level closely parallel to that of mouse passage virus, the differences observed might be attributed to a qualitative change in the virulence of individual virus particles, so that more would be required to produce a lesion equal in severity to that

TABLE IV

Comparison of Neutralizing Capacity of Same Sera Tested against Culture Virus of 54th Transfer and a New Culture in the 5th Transfer

Serum	Culture virus (P.R. 8)					
	54th transfer (Original culture)			5th transfer (New culture)		
	Mouse No.			Mouse No.		
	1	2	3	1	2	3
Normal rabbit 1-29	*++++	+++	+++	*++++	*++++	+
" swine 16-52	*++++	*++++	++++	*++++	*++++	+++
Swine 16-52, immune to swine influenza	*++++	+++	+++	++++	++	+++
Human R. D.	++±	++	+	+	++	++
" D. T.	+++	+++	+++	±+	++	++
" T. F.	++	++	++	++	++	++
" W. M.	0	0	+	0	0	0

produced by a smaller number of particles of virus maintained by animal passage. This possibility is heightened by the impression gained that the lesions in the mice infected with culture virus, although of equal extent, are less intense than those obtained with passage of the virus from animal to animal.

SUMMARY

The *in vitro* cultivation of strains of human influenza virus has been successfully conducted through a prolonged series of successive transfers. The cultivated virus has retained the antigenic and immunological properties which characterized the animal passage virus from

ON GROUP SPECIFIC A SUBSTANCES*

III. The Substance in Commercial Pepsin

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(Received for publication, February 26, 1936)

As mentioned in a previous paper (2), in the study of the substances underlying the serological groups of human beings the comparison of preparations from various sources appears desirable. In the present paper there is reported an investigation of the substance present in commercial pepsin which as first shown by Schiff and his colleagues (4–6) gives intense reactions with immune sera for human A erythrocytes. Indeed by this finding Schiff made available a source which permits obtaining without difficulty large amounts of a serologically highly active material. The literature has been reviewed before (2); it should be supplemented by mention of the investigation of Hallauer (3) on water soluble group specific substances from human erythrocytes or stromata, resulting in preparations containing carbohydrate and probably lipoid.

Preparation of the Substance.—A 2 per cent solution of pepsin (Fairchild Bros. and Foster, 1:15,000, made from pig stomachs) in 1 per cent saline, about pH 6 in reaction, was heated to 95° and kept in the steam bath for 10 minutes, then cooled and freed of coagulum by centrifugation. The supernatant fluid was acidified with 50 per cent acetic acid, 6 cc. per 100 cc. solution, and after being brought to 95°C. was heated for 5 minutes in the steam bath; it was then filtered hot through fluted paper. The filtrate was neutralized with NaOH and an equal volume of 95 per cent alcohol was added with stirring. After an hour or so at room temperature, with occasional stirring, the precipitate was removed by filtration.

The active substance was precipitated from the filtrate by adding one-quarter its volume of alcohol. The gummous precipitate either can be collected by centrifugation or can be allowed to settle. The sediment was rubbed up with water, 100 cc. for each 10 gm. of pepsin employed, and insoluble material was removed

* Previous communications (1, 2).

The substance was precipitable by tannic acid, by mercuric chloride, and by ammonium sulfate (4 volumes of saturated aqueous solution). Like the horse saliva substance, when the solution was strongly acidified it gave a heavy precipitate with phosphotungstic acid.

The preparation showed a strongly positive Elson and Morgan reaction. Colorimetrically, the content of amino sugar was determined[2] as 27 per cent; it was identified as glucosamine.

For identification of glucosamine, material was taken at a stage of purification prior to the Berkefeld filtration. It was sealed in a tube with N HCl and hydrolyzed in the steam bath, and the solution evaporated to dryness *in vacuo*; recrystallization was carried out in aqueous alcohol containing a small amount of HCl. From 500 mg. of substance, 140 mg. of hexosamine hydrochloride were obtained. It was converted into the anisal derivative (8), which melted at 165–166°C. (melting point of *p*-methoxybenzylidene *d*-glucosamine); there was no depression of the melting point on mixing with a known sample. Additional confirmation was obtained by determination of the initial and equilibrium rotations.

In testing for mucic acid by HNO_3 oxidation, 250 mg. of substance yielded 26.7 mg. of an insoluble acid melting at 220–221°C.; the presence of galactose was further confirmed by the isolation of the galactose-*o*-tolyl hydrazone from the products of H_2SO_4 hydrolysis.

In Tollens' test for uronic acids, the ether extract was colorless. Bial's orcin test for pentoses was negative, and the present substance in contrast to preparations from other sources gives no violet color (2) nor indeed any characteristic color with the reagent. The preparation gave a faintly positive reaction in the Sakaguchi test.

As regards elementary composition and the demonstration, after hydrolysis, of glucosamine and galactose, as well as the presence of acetyl groups (*cf.* Schiff, Freudenberg), the results reported are in conformity with the findings on the water soluble group specific A preparations from human urine[3] and horse saliva (9, 10, 2). A further point of agreement is the fact that the substance from pepsin, like the A substance in human A saliva and that separated from horse saliva, is decomposed by a carbohydrate-splitting bacterium (11).

[2] We are indebted to Dr. Karl Meyer of Columbia University for this determination.

[3] It may be interesting to note that at an early date a carbohydrate containing nitrogen was described in human urine by Salkowski (16).

by spinning. To 100 cc. of this solution 1 gm. of sodium acetate and 100 cc. of 95 per cent alcohol were added, and the fluid was passed through paper pulp to yield a perfectly clear filtrate, a new paper pulp bed being used for each 100 cc. portion. The precipitate separating upon addition to the filtrate of one-quarter its volume of alcohol was sedimented by centrifugation, washed with absolute alcohol, and dried.

The substance, which represented about 16 per cent of the starting material, still gave distinct xanthoprotein and ninhydrin reactions. To 100 cc. of a 2 per cent solution of this substance, which dissolves slowly in hot water, were added 1 gm. of sodium acetate and 110 cc. of 95 per cent alcohol. The turbid fluid was filtered through a large Berkefeld V candle, and if necessary refiltered until the solution was clear. Sufficient alcohol was added to raise the alcohol concentration to 57–60 per cent, and the precipitate was sedimented by centrifugation, washed with absolute alcohol and ether, and dried.

The material was then precipitated from 5 per cent solution by addition of glacial acetic acid until flocculation occurred; about 18 volumes were necessary. The precipitate, washed with absolute alcohol, dried, and finely ground, was freed of acetic acid by soaking in 70 per cent alcohol with frequent changes over a period of several days and was dried again. The yield was about 8–10 per cent of the commercial pepsin employed.

The substance dissolves slowly in water, yielding a slightly turbid, viscous solution which becomes clear when neutralized by a trace of alkali. Upon analysis the preparation was found to have: C 46.88 per cent, H 6.62 per cent, N 6.16 per cent; S 0.08 per cent; P 0.10 per cent; acetyl 9.95 per cent; ash, nil; reducing sugar following acid hydrolysis, as glucose, 70.7 per cent (mean value). The optical rotation determined from a 2.5 per cent aqueous solution was $[\alpha]_D^{30^\circ} = +16^\circ$.

At this stage of purification the preparation still gives, in 2 per cent solution, some weakly positive protein tests, as the biuret and xanthoprotein, and a faint turbidity with trichloroacetic acid. A distinct color reaction was obtained with diazotized sulfanilic acid.[1] The substance even in 5 per cent solution failed to give reactions with picric acid, lead acetate, sulfosalicylic acid, and uranyl nitrate, and the Millon and ninhydrin tests also were negative. As seen from the analysis, the preparation is practically free from sulfur.

[1] A similar instance is that reported by Rimington (7) concerning the isolation of the carbohydrate in horse serum where histidine causing a positive diazo reaction could be removed only by prolonged treatment with alkali.

A crude preparation of the A substance in beef pepsin was secured by heat coagulation, alcohol fractionation, and discarding inactive material by changes in hydrogen ion concentration; it was found to have 33 per cent reducing sugar, as glucose, after acid hydrolysis. The material proved to be strongly inhibitory in the customary hemolysis and isoagglutination tests (2) and also showed some degree of inhibition in the isoagglutination of human B cells.

The continuation of the investigation will chiefly involve attempts at further purification and more thorough chemical examination of the active substances.

The experiments were carried out with the assistance of Mr. Robert A. Harte.

SUMMARY

A method of preparation and a preliminary chemical investigation of the substance present in commercial (pig) pepsin which reacts with human A antiserum are presented. The material offers especial advantage in securing in quantity a serologically highly active preparation suitable for further studies. Active preparations were isolated moreover from commercial (pig) gastric mucin. Some other materials showing group specific reactions are mentioned.

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Of the substances named that from pepsin is peculiar in that it is precipitated by tannic acid and by mercuric chloride. Also it may be noted that the value for reducing sugar after hydrolysis is distinctly higher.

In hemolysis tests (2) the present substance was about as active as that from horse saliva, while it gave an even stronger inhibition of the isoagglutination of A cells.

A preparation very similar to that from commercial pepsin was obtained from a commercially available crude gastric mucin (pig)[4] which is rich in A substance, by removing insoluble material and fractionating with alcohol.

From the data at hand, it would appear that the A substances from human urine, horse saliva, and pig stomach, and the acetyl polysaccharide of Pneumococcus I which likewise reacts with A immune sera (13), all agree in that they possess amino sugar and galactose, and acetyl groups most probably on the amino group of the hexosamine. Whether both acetylated hexosamine and galactose are essential for the specificity of the reaction remains to be determined. However, in view of the observations of Freudenberg on human urine of group O (and group B) it seems that polysaccharides which contain these constituents need not react with anti-A sera.

Although the A preparations so far examined cannot with certainty be regarded as chemical individuals, yet on account of the differences observed, particularly the apparent differences in serological activity, it is possible that the active substances are not identical, and that the reaction with A immune serum is a group reaction exhibited by various substances which have certain structural similarities, as doubtless is the case with the Forssman antigens.

Substances reacting to a greater or lesser degree with A immune sera seem to be rather widely distributed; such reactions we found with gastric juice of the dog, with rabbit stomach mucosa, with a substance found in commercial beef pepsin (Armour and Company), and with extracts of *N. catarrhalis*,[5] although here the effect of constituents of the medium is not entirely excluded. In this connection the observations on anti-A agglutinins in antibacterial sera (paratyphoid B (14, 15)) may be mentioned.

[4] Gastric mucin, No. 1701-W (Wilson and Co.); see Miller (12).

[5] This culture was supplied through the courtesy of Dr. G. Howard Bailey.

QUANTITATIVE STUDIES ON THE PRECIPITIN REACTION

Effect of Salts on the Reaction*

By MICHAEL HEIDELBERGER, Ph.D., FORREST E. KENDALL, Ph.D., and TORSTEN TEORELL,** M.D.

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(Received for publication, March 20, 1936)

In the studies on the precipitin reaction hitherto published from this laboratory (1) the salt concentration was held constant at 0.9 per cent of sodium chloride in order to permit observations on the effect of varying the proportions of antigen (or hapten) and antibody. In the present paper are reported data obtained during several years on the influence on the course of the precipitin reaction of changes in the concentration of sodium chloride and on the effect of other cations and anions.

According to our quantitative theory of the precipitin reaction, expressions of the form,

$$\text{mg. antibody N precipitated} = 2RS - \frac{R^2S^2}{A}$$

may be used to describe the course of the precipitin reaction (2). In these equations S refers to the specific polysaccharide or antigen, R to the ratio between antibody nitrogen and S precipitated at a definite reference point in the "equivalence zone" (2), and A to the antibody nitrogen precipitated at the reference point. The effect of increased salt concentration on these equations is also discussed in the present paper.

EXPERIMENTAL

The analytical technique used was that described in previous papers of the series (1–3). In the experiments summarized in Table I the final concentration

* The work reported in this communication was carried out under the Harkness Research Fund of the Presbyterian Hospital.

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TABLE I

Effect of the Concentration of Sodium Chloride upon the Reaction between S III and Antibody. 37° and 0°

Final NaCl concentration	Horse antibody solution B 36					Rabbit antibody solution B 50*	
	0.1 M	0.15 M	0.51 M	0.93 M	1.79 M	0.15 M	0.93-0.93 M
S III used	Nitrogen precipitated						
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
0.02	0.54	0.50	0.42	0.39	0.36		
0.05	1.13	1.03	0.90	0.84	0.75	0.43	0.24
0.075	1.41	1.41	1.29	1.15	1.03	0.60	
0.10	1.75	1.66	1.54	1.28	1.22	0.77	0.34
0.15	1.78	1.86	1.62	1.50	1.45	1.04	0.39
0.20†	1.82	1.85	1.70	1.58	1.51	1.18	0.41
Equations:‡							
mg. antibody N pptd.	27.5 S–104 S^2	25 S–84 S^2	22.2 S–72 S^2	20.2 S–68 S^2	18.1 S–57 S^2	9.5 S–18 S^2	5.0 S–15 S^2
A§	1.82	1.86	1.71	1.50	1.44	1.25	0.42

* Prepared according to Felton (5).

† Excess S III.

‡ *Cf.* Reference 2.

§ Calculated mg. antibody N pptd. at antibody excess end of equivalence zone.

of salt was varied from 0.6 per cent to 10.45 per cent, or 0.1 M to 1.79 M. 1.0 ml. of antibody solution B 36, prepared according to Felton (4) and made up with 0.9 per cent saline, was mixed in each case with the given amount of the specific polysaccharide of Type III pneumococcus (S III) dissolved in 3 ml. of salt solution of such strength that the final concentration of salt was as indicated in the table. The experiments were run for 2 hours at 37° and overnight at 0°, so that the results do not represent the maximum amount of antibody precipitable by any of the quantities of S III used (3). However, the amount of antibody nitrogen precipitable by a given quantity of S III decreased with increasing salt concentration, as did also the total amount of antibody nitrogen precipitable.

In Table II are given results of experiments run separately at 0° and at 37° in 0.9 per cent and 10 per cent sodium chloride solutions, using S III and Type III antipneumococcus horse serum 607 which had previously been precipitated with C substance and pneumococcus protein. 1.0 ml. of antiserum was added to a mixture of 1.0 ml. of S III solution in water and 2.0 ml. of salt solution of such strength as to give the desired final concentration. The tubes run at 0° were allowed to stand for 48 hours, except those containing excess S III, for which 4 days were allowed, while those at 37° were centrifuged after 2 hours, experiments having shown no significant differences between the amounts of antibody precipitated at 37° in 0.5, 1, 2, 4, or 6 hours.

Table III summarizes the data obtained with a divalent cation and divalent and tetravalent anions in the S III-antibody system. In this series of experiments 3.0 ml. of stock salt solution, 1.5 ml. of S III solution in water, and 1.5 ml. of antibody solution B 65 were mixed.

Table IV deals with the effect of salts on the reaction between crystalline egg albumin (Ea) and rabbit anti-egg albumin serum.

DISCUSSION

The data summarized in Table I show that at five different sodium chloride concentrations ranging from 0.1 to 1.79 molar, the amount of antibody nitrogen precipitated by a given quantity of S III from homologous antibody solution decreases with increasing salt concentration. This progression is reflected in a decrease in both constants of the equation for antibody precipitated, derived according to Reference 2. A similar effect is shown in whole serum both at 0° and at 37° (Table II), and it will be noted that the differences between the amounts of antibody precipitated from 0.15 and 1.75 M salt solution are approximately the same at both temperatures.

In an attempt to find the reason for the decrease in the amount of nitrogen precipitated at higher salt concentration the supernatants from the precipitate formed by the interaction of 0.07 mg. of S III and

TABLE I

Effect of the Concentration of Sodium Chloride upon the Reaction between S III and Antibody. 37° and 0°

Final NaCl concentration	Horse antibody solution B 36					Rabbit antibody solution B 50*	
	0.1 M	0.15 M	0.51 M	0.93 M	1.79 M	0.15 M	0.93–0.93 M
S III used	Nitrogen precipitated						
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
0.02	0.54	0.50	0.42	0.39	0.36		
0.05	1.13	1.03	0.90	0.84	0.75	0.43	0.24
0.075	1.41	1.41	1.29	1.15	1.03	0.60	
0.10	1.75	1.66	1.54	1.28	1.22	0.77	0.34
0.15	1.78	1.86	1.62	1.50	1.45	1.04	0.39
0.20†	1.82	1.85	1.70	1.58	1.51	1.18	0.41
Equations:‡ mg. antibody N pptd.	27.5 S–104 S^2	25 S–84 S^2	22.2 S–72 S^2	20.2 S–68 S^2	18.1 S–57 S^2	9.5 S–18 S^2	5.0 S–15 S^2
A§	1.82	1.86	1.71	1.50	1.44	1.25	0.42

* Prepared according to Felton (5).

† Excess S III.

‡ *Cf.* Reference 2.

§ Calculated mg. antibody N pptd. at antibody excess end of equivalence zone.

of salt was varied from 0.6 per cent to 10.45 per cent, or 0.1 M to 1.79 M. 1.0 ml. of antibody solution B 36, prepared according to Felton (4) and made up with 0.9 per cent saline, was mixed in each case with the given amount of the specific polysaccharide of Type III pneumococcus (S III) dissolved in 3 ml. of salt solution of such strength that the final concentration of salt was as indicated in the table. The experiments were run for 2 hours at 37° and overnight at 0°, so that the results do not represent the maximum amount of antibody precipitable by any of the quantities of S III used (3). However, the amount of antibody nitrogen precipitable by a given quantity of S III decreased with increasing salt concentration, as did also the total amount of antibody nitrogen precipitable.

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Table III summarizes the data obtained with a divalent cation and divalent and tetravalent anions in the S III-antibody system. In this series of experiments 3.0 ml. of stock salt solution, 1.5 ml. of S III solution in water, and 1.5 ml. of antibody solution B 65 were mixed.

Table IV deals with the effect of salts on the reaction between crystalline egg albumin (Ea) and rabbit anti-egg albumin serum.

DISCUSSION

The data summarized in Table I show that at five different sodium chloride concentrations ranging from 0.1 to 1.79 molar, the amount of antibody nitrogen precipitated by a given quantity of S III from homologous antibody solution decreases with increasing salt concentration. This progression is reflected in a decrease in both constants of the equation for antibody precipitated, derived according to Reference 2. A similar effect is shown in whole serum both at 0° and at 37° (Table II), and it will be noted that the differences between the amounts of antibody precipitated from 0.15 and 1.75 M salt solution are approximately the same at both temperatures.

In an attempt to find the reason for the decrease in the amount of nitrogen precipitated at higher salt concentration the supernatants from the precipitate formed by the interaction of 0.07 mg. of S III and

trations is not due to increased solubility of the specific precipitate, but rather to a shift in the equilibrium brought about by the presence of the salt, by which the same amount of S III combines with a smaller quantity of antibody. This point will be discussed below in greater detail.

The data summarized in Table III show no very clear effect of a divalent cation or anion on the final result other than that of the total ion concentration, unless the approximately equal influence of 0.51

TABLE III

Effect of Varying Cations and Anions on Reaction between S III and Antibody Solution B 65

S III used	Antibody N precipitated				
	0.15 M NaCl	0.51 M NaCl	0.31 M $MgCl_2$	0.46 M Na_2SO_4	0.35 M $K_4Fe(CN)_6$
mg.	mg.	mg.	mg.	mg.	mg.
			Experiments at 0°		
0.020	0.43	0.39	0.34	0.35	0.40
0.045	0.76	0.58	0.57	0.61	0.68
0.150*	0.71	0.65	0.65	0.66	0.76
			Experiments at 37°		
0.020	0.40		0.27		
0.045	0.68	0.45	0.47†	0.55†	0.62
0.150*	0.72	0.59	0.62		0.62†

0.51 M NaCl, 0.31 M $MgCl_2$, 0.46 M Na_2SO_4, and 0.35 M $K_4Fe(CN)_6$ were calculated to give approximately the same osmotic pressure, equivalent to 1.8° depression of the freezing point of water.

* Excess S.

† One determination.

molar sodium chloride and 0.31 molar magnesium chloride ($MgCl_2$) be ascribed to the cation effect alone. The tetravalent ferrocyanide ion definitely counteracted the salt concentration effect. In addition it was observed that the magnesium ion decreased the flocculation velocity of the specific precipitate while the anions of higher valence increased the velocity, even in the zone of partial inhibition.

From Table IV it will be seen that the egg albumin-antibody reaction was found relatively insensitive to variations in salt concentration, contrary to the findings of Downs and Gottlieb (7) in qualitative

serum 607 in 1.75 M salt solution (Table II) were combined and dialyzed at 0° against 0.15 M salt solution. According to the equation in column 2, Table II, 0.07 mg. of S III should precipitate 1.08 mg. of antibody N in 0.15 M salt solution at 0°. Thus, if the effect in 1.75 M salt solution were one of solubility, the decrease in salt concentration

TABLE II

Effect of Varying Sodium Chloride Concentrations on Reaction between S III and Antiserum 607 at 0° and at 37°

S III used	Antibody N precipitated at 0°		Antibody N precipitated at 37°	
	0.15 M NaCl	1.75 M NaCl	0.15 M NaCl	1.75 M NaCl
mg.	*mg.*	*mg.*	*mg.*	*mg.*
0.03		0.48		0.42
0.04	0.74		0.64	
0.07		0.83*		0.78
0.075	1.12	0.91	0.97	
0.10	1.24	1.01	1.09	0.87
0.15†		1.07		0.91
0.30†		1.10		0.94‡
1.00†		1.08		0.85
2.34†		0.87		0.54
Equations:§				
mg. antibody N pptd.....	22.5 S–101 S^2	18.4 S–83 S^2	19.3 S–85 S^2	16.2 S–75 S^2
A‖.....	1.25	1.02	1.10	0.87

* The supernatant remained clear when dialyzed at 0° against 0.9 per cent saline. The nitrogen determinations recorded in Tables II, III, and IV were run according to Teorell (6).

† Excess S.

‡ One determination.

§ *Cf.* Reference 2.

‖ Calculated mg. antibody N pptd. at antibody excess end of equivalence zone.

due to the dialysis should have caused 1.08–0.83, or 0.25 mg. of antibody nitrogen to precipitate for each of the supernatants which were combined and dialyzed. However, the solution remained clear, although it still contained antibody N which precipitated when more S III was added after the dialysis. It is therefore probable that the decreased amount of antibody precipitated at the higher salt concen-

back of the ionization of the soluble antibody-sodium chloride complex (*cf.* 11) is difficult to say. It is also possible that the large Coulomb forces due to the heaping up of carboxyl groups in the S III molecule (*cf.* 12) result in a spatial configuration which is altered when these charges are reduced by high salt concentrations, with a consequent diminution in specificity, or reactivity with corresponding spatially orientated groupings in the antibody molecule. At any rate there is much reason to ascribe an ionic mechanism to this effect, as was originally done in the case of the S III-antibody reaction itself (10). Salt effects similar to the above have also been noted by Hammarsten and collaborators (13), in the reaction between proteins and nucleic acids.

If the effect of high concentrations of salts on the precipitin reaction consists of a shift in the S III-antibody equilibrium, and if this shift is reversible, a method is available by which, with strong salt solutions, it should theoretically be possible to dissociate pure antibody from a specific precipitate formed at 0.9 per cent salt concentration in a suitable region of the reaction range. This phase of the work will be discussed in a paper now in preparation.

SUMMARY

1. A quantitative study has been made of the effect on the precipitin reaction between the specific polysaccharide of Type III pneumococcus and the homologous antibody of salt concentrations ranging from 0.1 M to 1.79 M, including the effect of ions of higher valence.

2. Within these limits, observed decreases in precipitated antibody with increasing salt concentration appear to be due to a decrease in the amount of antibody combined with the S III, rather than to an increase in solubility of the S III-antibody compounds.

3. The egg albumin-antibody reaction is far less sensitive to changes in salt concentration than is the S III-antibody reaction.

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experiments with molar solutions, including sodium chloride, and with ferrocyanide.

In general, it may be said that the present observations on the effect of salts on the precipitin reaction are probably compatible with any of the theories which have been proposed for this reaction and are certainly not incompatible with our quantitative theory (1, 2, 8). It is not possible, however, to give a quantitative interpretation of the salt effects with the data at hand or with the methods which proved adequate at physiological salt concentrations. For example, it is possible

TABLE IV

Effect of Varying Salt Concentrations on the Precipitin Reaction between Egg Albumin (Ea) and Homologous Antibody, Serum 387 III, 1:1, at 0°*

Ea N used	Total mg. N precipitated					
	Cation effect				Anion effect	
	0.15 M NaCl	0.51 M NaCl	1.75 M NaCl	0.37 M $MgCl_2$	0.46 M Na_2SO_4	0.35 M $K_4Fe(CN)_6$
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
0.030	0.67	0.61		(0.53)	0.63	0.63
0.079	1.32	1.32	1.32	1.28	1.32	1.34
0.296†	1.03	−‡		−‡	0.95	1.09

* *Cf.* Reference 1.

† Excess Ea.

‡ The milky suspension deposited very little solid when centrifuged under the usual conditions.

that the opalescent solutions obtained in certain instances in the inhibition zone would have yielded more precipitate if centrifuged at higher speed. The electrolyte content of the precipitates is also unknown.

While the observed salt effects show certain similarities to those noted with typical globulins (9), the dialysis experiment quoted above (Table II) indicates that the diminution in precipitable nitrogen is not due to increased solubility of the specific precipitate. The cause appears to be rather a shift in the proportions in which S III and antibody combine. Whether this is due to a competition of the cation with antibody for combination with the S III anion (*cf.* 10), or a driving

EXPERIMENTAL ENCEPHALITIS (ST. LOUIS TYPE) IN MICE WITH HIGH INBORN RESISTANCE

A Chronic Subclinical Infection

By LESLIE T. WEBSTER, M.D., and ANNA D. CLOW

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

Plates 48 to 50

(Received for publication, February 26, 1936)

Previously we have described how the St. Louis encephalitis virus dropped into the nares of highly susceptible mice follows the route of the olfactory nerves to reach the brain within 24 hours. There it incites lesions within 3 days, multiplies rapidly, and sets up a fulminating encephalitis clinically apparent on the 6th day and fatal by the 10th day (1). These mice were generally uniform in their response to the virus.

Attention was next turned to highly resistant mice of the same stock, in fact, originating from the same parents as the susceptibles, and likewise generally uniform in their response to the virus. In these mice virus instilled nasally, although following the olfactory nerve route and reaching the brain promptly, gives rise there to an infection which is non-fatal, subclinical, and chronic. These findings and their possible implications are described in the present paper.

Materials

The resistant mice used in these studies were derived from the same hybrid stock as the susceptibles (2). In brief, two litters from the same parents were mated brother to sister. From the one litter-mating, the virus-susceptible line was developed; from the other, the virus-resistant line[1] (Text-fig. 1).

The susceptibles in general succumb to an intracerebral dose of 10^{-9} and to an intranasal dose of 10^{-6} gm. of mouse brain virus, while the resistants succumb to an intracerebral dose of 10^{-5} or greater and survive for the most part an intranasal dose of 10^{-3} gm. By either route, therefore, the resistants survive at

[1] These lines and their development will be described more fully in a forthcoming paper.

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This quantity is about ten lethal doses for susceptibles and is non-fatal for resistants. At the same time, eight additional resistant mice received 10,000 times this amount, intracerebrally,—0.03 cc. of a 10^{-2} dilution. At 6 hours and 3, 6, 9, 13, and 21 days after injection, one animal from each group was sacrificed and its blood, spleen, and various portions of its brain were tested for the presence of virus. The materials for testing were emulsified, prepared in serial, tenfold dilutions, and injected intracerebrally in 0.03 cc. quantities into two Swiss mice. The titre of virus in the test material was taken as the highest dilution fatal to at least one of the two injected mice expressed in numbers of intracerebral lethal doses on the basis that 0.03 cc. of 10^{-7} dilution is one fatal dose. The fully virulent brain, therefore, is said to contain 0.03×10^{7} fatal doses, or roughly 10^{9}. The titre is taken as the exponential value of the highest fatal dilution with sign changed increased by 2,—10^{-7} dilution, or 10^{9} titre.

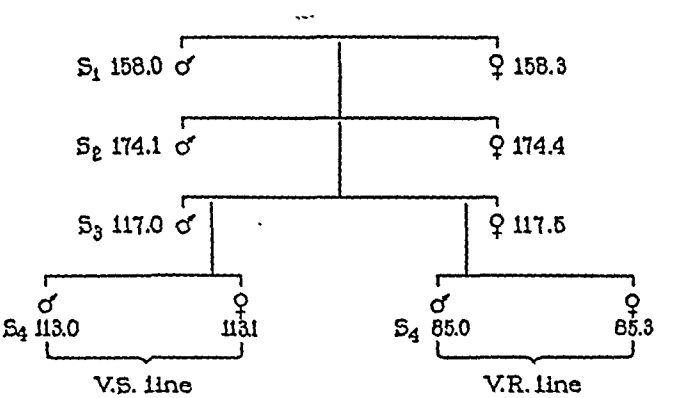
The results are given in Table I. No virus was recovered from the susceptible mouse sacrificed at 6 hours. From the one examined at 3 days, virus was recovered in blood, spleen, olfactory areas, and in the remainder of the brain in 10^{-2} dilution, while from the one tested in convulsions on the 6th day, virus was found in blood and spleen and in whole brain diluted 10^{-7} times. The remaining five susceptibles died of encephalitis without being tested on the 5th and 6th days. All resistant mice receiving a like amount of virus remained well. At 6 hours virus was not recovered, at 3 days only from the piriform area, and at 9 days from olfactory areas and the remainder of the brain in 10^{-2} dilution. No virus was found at 13 and 21 days. Resistant mice receiving the large dose remained well, save for one dying on the 13th day, but showed virus in blood, spleen, olfactory areas, and the remainder of the brain in 10^{-2} dilution at 6 hours, spleen and olfactory areas at 3 days, olfactory areas on the 6th day, and in the remainder of the brain, besides, on the 9th day. On the 13th day, no virus was recovered.

Injected intraperitoneally, the virus behaves similarly in both sorts of mice. The following experiment illustrates the happenings.

Experiment 2.—Mar. 27, 1934. Resistant mice were given 0.5 cc. of a 1 to 200 dilution of virus intraperitoneally. At intervals from 10 minutes to 7 days after injection, individuals were sacrificed and their blood, brains, and spleens tested for the presence of virus, according to the technique previously described. An equal number of susceptible mice was given the intraperitoneal injection for comparison and three from each group were reserved as controls.

least 1,000 fatal doses for susceptibles. Mice of a given strain are not, however, entirely uniform in their response to a given exposure in spite of brother to sister inbreeding for twelve generations and standardization of environment. In comparing strains by means of a nasal instillation of 100 to 1,000 nasal lethal doses of virus for susceptibles, about 95 per cent of the susceptibles will die, as contrasted with 15 per cent of the resistants. Consequently a departure from expectancy of about 10 per cent was allowed for whenever indicated. We have for comparison, then, two lines of progeny from sibling litters, one line transmitting virus-susceptible, the other virus-resistant factors.

The test virus was strain 3 (1) passed as routine intracerebrally in susceptible mice.



TEXT-FIG. 1. *Mus musculus albinus*, Rockefeller Institute strain. Lineage of virus-susceptible and resistant lines from brother-sister matings of two sibling litters.

Limited Neurotropism of Virus in Resistant Mice

In resistant mice, virus shows the same sort of predilection for nervous tissue as in susceptible mice (1). Injected in maximum doses intracerebrally in resistants, its titre increases following an initial lag and lesions arise in the brain in the same manner as in susceptibles. Blood is contaminated immediately following injection and preceding death, and other organs are free of lesions (1). Small doses fatal to susceptibles but not to resistants bring about localized brain lesions in the latter and the virus persists but does not increase beyond a certain level. These findings are partly illustrated in the following experiment.

Experiment 1.—Jan. 14, 1936. Eight susceptible and eight resistant mice were each given 0.03 cc. of a 10^{-6} dilution of mouse brain virus intracerebrally.

The controls remained well. Virus in the resistant mice was distributed in about the same manner as in the susceptible mice. 10 minutes after injection it was present in the spleen and at 20 and 60 minutes in the undiluted blood as well. It was absent from the blood at 3 hours and at all intervals tested thereafter. The spleens, however, contained virus at 3 and 9 hours and at 1, 2, and 3 days. Further tests showed that virus reached the blood as quickly in resistant as in susceptible mice but in somewhat less amounts and for shorter periods. Spleens and brains showed no lesions 2, 5, and 10 days following injection of virus.

Chronic Infections Following Nasal Instillation of Virus

The tests thus far described showed that resistants withstood many intracerebral fatal doses for susceptibles, that virus persisted at low titre for about 9 days and then disappeared, and that lesions following injection cleared up progressively. When it came to instilling the virus intranasally into resistant mice, the following distinguishing features were noted,—first, 1,000 intranasal doses fatal for susceptibles were not harmful to resistants; second, the titre of virus in the brain did not increase beyond a certain point; third, virus persisted in the brains as long as 4 weeks; fourth, lesions in the brain did not appear for 8 days but once present, were found for 3 months, while the animal remained quite well; fifth, these lesions closely resemble those found in human cases of encephalitis. These findings are described in the following experiments.

Intranasal Inoculation. Experiment 3.—Dec. 27, 1934. Batches of resistant and susceptible mice were given a nasal instillation of 0.03 cc. of a 1 to 100 dilution of virus. At intervals from 1 to 21 days after injection animals were sacrificed and their brains and spleens tested for content of virus as in Experiment 1. Twelve resistant and five susceptible mice were reserved as controls.

The results are shown in Table II. The susceptible controls died in 6 and 7 days; one resistant control died on the 9th day. No virus was found in brains or spleens of resistant or susceptible mice on the 1st day following injection. On the 2nd day, however, virus was present in brains of both resistants and susceptibles in 10^{-1} and 10^{-2} dilutions, respectively. On the 3rd day, virus was present in resistants in 10^{-1} and 10^{-2} dilutions, as contrasted with 10^{-4} and 10^{-6} in suscep-

TABLE I

Distribution and Titre of St. Louis Virus in Susceptible and Resistant Mice Following Intracerebral Injection

Type of mouse	Dose of virus 0.03 cc. of dilution	Time after injection	Presence of virus. 0.03 cc. of dilution to two mice									
			Blood	Olfactory bulb	Piriform area	Remainder of brain						Spleen
			Undiluted	10^{-1}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
Susceptible	10^{-5}	6 hrs.						N.T.		N.T.	N.T.	
"	10^{-6}	3 days	7,* 8	7, 7	6, 8	8, 8	N.T.	"		"	"	6, 7
"	10^{-6}	†6 "	6, 7	N.T.	N.T.	N.T.	"	"	N.T.	6, 6	7, 7	7, 7
Resistant	10^{-5}	6 hrs.						N.T.		N.T.	N.T.	
"		3 days			7, 8		N.T.	"		"	"	
"		6 "					"		N.T.	"	"	
"		9 "		8, 10	7, 8	8	"		"	"	"	
"		13 "										
"		21 "										
Resistant	10^{-2}	6 hrs.	7, 8	8	8	8, 8	N.T.		N.T.	N.T.	N.T.	7
"		3 days		7, 7	6		"		"	"	"	7, 8
"		6 "		7	7	9	"		"	"	"	
"		9 "			9, 9	6, 8	"	8, 9				
		13 "										

* = duration of life of mouse in days.

N.T. = dilution not tested.

Blank spaces = mice remained well 21 days.

† Mouse in convulsions.

tibles. On the 4th, 5th, 6th, 7th, and 12th days, virus in the brains of resistant mice remained at the 10^{-1} to 10^{-3} dilution level, while in the susceptibles it reached a maximum tested of the 10^{-7} dilution on the 6th day. By the 7th day all susceptibles had died of encephalitis. The batch of resistants for testing remained well. At 18 and 21 days no virus was found in their brains. Spleens were irregularly positive in both groups from the 3rd to the 7th days inclusive.

Additional experiments on resistant mice gave results similar to the above together with one brain of ten positive at 4 weeks and with negative findings in the blood from 1 hour to 7 days after nasal instillation of virus. Moreover, spleens of twenty-one healthy, resistant mice were tested 4 to 5 weeks following nasal instillation of virus with four, or 19 per cent, positive and spleens of thirty-one mice after 6 weeks with four, or 13 per cent, positive.

Nasally infected, resistant mice were tested further with relation to the distribution of virus in various portions of the brain.

Experiment 4.—Jan. 27, 1936. A. Eight resistant mice received intranasally 0.03 cc. of a 1 to 100 dilution of virus of tested standard titre, 10^9. At intervals thereafter of 1 to 11 days animals were sacrificed, their brains removed, sectioned, and tested for content of virus. Olfactory bulbs, piriform area, cortex, cerebellum, pons, and medulla were each emulsified with alundum, diluted roughly one part to ten of hormone broth, and injected intracerebrally into two Swiss mice.

Jan. 31, 1936. B. Seven additional resistant mice were given virus in the same manner and sectioned for content of virus on the 7th, 10th, 14th, 16th, 18th, 21st, and 23rd days following instillation.

At the same time additional susceptibles and resistants were given virus intranasally, sacrificed at the stated intervals, their brains fixed in Zenker's acetic solution, sectioned, and stained for histological study. Reference to this material is made later.

The distribution of virus in these mice is shown in Table III. At 24 hours it was present in the olfactory bulbs. At 2 days and in one mouse at 3 days, no virus was recovered.

In the second mouse at 3 days and in the one mouse examined at 5 days, virus was recovered from olfactory bulbs, piriform area, and pons. At 7, 9, 10 (one mouse), and 11 days, it was present in all regions tested. At 13, 15, 18, and 21 days, no virus was recovered.

Evidently, therefore, virus traverses from nose to olfactory bulbs in resistant mice as promptly as in susceptible mice and spreads

TABLE II

Brain and Spleen Content of St. Louis Virus in Resistant and Susceptible Mice Following Nasal Instillation

Mice		Time interval injection to test	Content of virus. 0.03 cc. of each dilution to two mice							
			Brain							Spleen
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-1}
		days								
Resistant	1	1			N.T.	N.T.	N.T.	N.T.	N.T.	
	2	1			"	"	"	"	"	
	3	2	5,* 5			"	"	"	"	
	4	2	5, 5			"	"	"	"	
	5	3	5, 8			"	"	"	"	
	6	3	5, 6	6, 6		"	"	"	"	6
	7	4	5, 7	7, 7		"	"	"	"	6, 7
	8	4	5, 7	8		"	"	"	"	6, 7
	9	5	6, 6	6, 7	6, 6		"	"	"	6, 6
	10	5	6				"	"	"	
	11	6	6, 6	6, 7	6, 8			"	"	
	12	6	6, 6	6, 6	6, 8			"	"	
	13	7	6, 6	6, 7				"	"	
	14	7	5, 5	6, 6				"	"	6, 7
	15	12	6, 6	6, 7	6		"	"	"	
	16	12	6, 6	7, 8			"	"	"	
	17	18				"	"	"	"	
	18	18				"	"	"	"	
	19	21				"	"	"	"	
	20	21				"	"	"	"	
	21	21				"	"	"	"	
Susceptible	1	1			N.T.	N.T.	N.T.	N.T.	N.T.	
	2	1			"	"	"	"	"	
	3	2	5, 6	5		"	"	"	"	
	4	2	6, 9			"	"	"	"	
	5	3	5, 5	5, 5	5, 6	6, 8	6, 9	6, 9	"	7, 8
	6	3	5, 6	6, 6	6, 6	9, 10			"	
	7	4	N.T.	5, 5	8, 8				"	
	8	4	"	5, 5	5, 7	5, 7	8, 8	8	"	
	9	5	"	4, 6	6, 6	6, 8	7, 7	7, 7	"	5, 5
	10	5	"	4, 4	6, 6	6, 6	6, 7	6, 6	"	
	11	6	"	N.T.	5, 5	5, 5	5, 5	5, 6	6, 6	4, 4
	12	6	"	"	5, 6	7, 8	7, 8	8, 8		6, 8
	13	7	"	"	5, 6	6, 7	7, 7	7, 8	8, 8	8, 8
	14	7	"	"	5, 5	5, 5	5, 5	5, 5	7, 7	8, 8

* = duration of life of mouse in days.

N.T. = dilution not tested.

Blank spaces = mice remained well 21 days.

and ten each of piriform area, anterior cerebrum, posterior cerebrum, mid-brain, cerebellum, and medulla, cervical, thoracic, and lumbar cord. The sections, 5 to 10μ in thickness, were mounted in strips of four to ten per slide, and stained for the most part with eosin-methylene blue. Virulence of the virus was checked by intracerebral and intranasal titrations in susceptible mice. All resistant mice remained well.

3 Days after Nasal Instillation.—Brains of two tested mice contained virus in the 10^{-2} dilution (Table IV). The sections of brains of three mice appeared normal.

TABLE IV

Brain Content of St. Louis Virus in Resistant Mice Following Intranasal Instillation

Mice	Time interval injection to test	Brain content of virus. 0.03 cc. of each dilution to two mice			
		10^{-1}	10^{-2}	10^{-3}	10^{-4}
	days				
Resistant 1	3	6,* 7	8, 10		
2	3	8, 8	9		
3	6	6, 6	6, 8	8, 9	10
4	6	6, 8	6, 9	10	
5	8	6, 7			N.T.
6	8	9			"
7	10	7, 7	8, 9		"
8	10	5, 5	7, 8		"
9	15	6			"
10	15	6			"
11	20			N.T.	"
12	20			"	"

* = duration of life of mouse in days.

N.T. = dilution not tested.

Blank spaces = mice remained well 21 days.

6 Days after Nasal Instillation.—Brains of the two tested mice showed virus in the 10^{-3} and 10^{-4} dilutions respectively, but the sections of brains of the three mice examined for lesions showed nothing abnormal.

8 Days after Nasal Instillation.—Brains of two mice tested at this time were positive only in the 10^{-1} dilution. Of three mice examined for lesions in the brain, one was negative and two showed inflammatory changes in the ventral, medial portions of the olfactory bulbs and piriform lobes similar in every respect to the primary lesion seen in susceptible mice on the 3rd day (1). Collected in the perivascular or subpial spaces, or scattered superficially nearby, were round cells and occasional polymorphonuclear leucocytes (Fig. 1). Small blood vessels in the vicinity were congested but all nerve cells appeared normal.

throughout the brain. The striking difference lies in its failure to multiply readily in resistants and its ability to survive for periods as long as 3 to 4 weeks.

Other events in the intranasally instilled mice were the late appearance, long duration, and character of central nervous system lesions.

TABLE III

Distribution of St. Louis Virus in Brains of Resistant Mice Following Nasal Instillation

Mouse No.	Time interval injection to test	Content of virus. 0.03 cc. of 1 to 10 dilution injected intracerebrally into two mice					
		Olfactory bulbs	Piriform area	Cortex	Pons	Cerebellum	Medulla
	days						
Resistant 1A	1	7,* 7					
2A	2						
3A	3						
4A	3	6	9		9		
5A	5	5, 7			7		
6A	7	6, 6	6, 6	8, 8	6, 6	6, 8	6, 8
7A	9	5, 7	5, 6	5, 7	6, 7	6, 6	6, 7
8A	11	D, 6	6, 6	7, 7	6, 6	7, 7	7, 7
Resistant 1B	10	7, 7	8, 9	7, 7	7, 7	7, 8	8
2B	10						
3B	13						
4B	13						
5B	15						
6B	18						
7B	21						

* = duration of life of mouse in days.

N.T. = dilution not tested.

Blank spaces = mice remained well 21 days.

D = mouse died of trauma following injection.

Lesions Following Nasal Instillation of Virus

Experiment 5.—Mar. 26, 1935. Resistant mice were given an intranasal instillation of 0.03 cc. of a 1 to 100 dilution of virus. On the 3rd, 6th, 8th, 10th, 15th, and 20th days following injection, two mice were sacrificed and the virus content of their brains titred in the usual manner. Three additional mice were sacrificed at each time and their brains studied histologically for the presence of lesions. From the brain of each mouse about fifty sections were prepared of olfactory bulbs

neighboring blood vessels. Another type of lesion became conspicuous, however, namely, collections of round cells in the Virchow-Robin spaces of vessels deep in the caudate nucleus and in neighboring areas, associated with glial cells and one or two degenerating nerve cells (Figs. 5 and 6). This type of lesion, so characteristic of the human disease and of lethargic encephalitis as well, became most marked at about 5 weeks, shortly after active virus could no longer be recovered. At the same time other vessels scattered through the cortex, pons, and medulla showed perivascular cuffs of round cells. At 51 to 54 days, the same lesions were present but fewer in number.

At 72 to 77 days, four of ten mice examined showed the above changes but still less extensive. At 97 days, one of three brains showed a few round cells beneath the pia and near neighboring vessels. At 117 days three of eight mice showed a similar slight exudate of leucocytes.

Similar studies on additional batches of resistant mice (Experiment 4) confirmed the above results.

In brief, resistant mice given virus intranasally remained well but showed brain lesions from 8 to 117 days after infection, while virus was recovered from 1 to about 28 days (Text-fig. 2). Lesions at first consisted of an exudate of mononuclear plus a few polynuclear leucocytes beneath the ventral pia of the olfactory bulbs and piriform area and in the Virchow-Robin spaces surrounding neighboring blood vessels. In some instances the pia was hyperplastic. In two cases only, examined on the 10th and 15th days, was nerve cell necrosis conspicuous. After 3 weeks, leucocytes were found deeper in the brain substance, collected about blood vessels in the cortex and medulla and in foci especially in the caudate nucleus, accompanied by glial cells and occasionally by a necrotic nerve cell. After 8 to 12 weeks, lesions became less apparent, some brains appeared normal, while others showed only an occasional collared vessel deep in the caudate nucleus region or a few leucocytes beneath the pia.

These changes, unlike those in susceptible mice (1), resemble closely those in the human disease and in lethargic encephalitis as well, and certain cases of atypical encephalitis and bulbar poliomyelitis.

Alteration in Amount of Virus by Passage through Resistant Mice

The routine virus is altered by passage through resistant mice.

Experiment 7.—Nov. 18, 1935. Ten virus-resistant mice were given a nasal instillation of 0.03 cc. of a 1 to 100 dilution of freshly prepared mouse brain virus as described in Experiment 3. At intervals thereafter, from 1 to 7 days, mice

10 Days.—Virus was present in the brains of the two tested mice through th 10^{-2} dilution.

All mice appeared well and one of the three examined for brain lesions showed nothing abnormal. In the second mouse the inflammatory lesion was marked on the ventral aspect of olfactory bulb and piriform areas (Fig. 2). Leucocytes were collected in considerable numbers about the superficial blood vessels and were scattered throughout the neighboring tissue. They were also present around the deep blood vessels of the cortex. Hyperplasia of the pia, noted in susceptible mice on the 6th day, was conspicuous over the ventral, anterior piriform area. The third mouse showed these lesions in a more advanced state, plus necrosis of a few pyramidal nerve cells in the ventral piriform area. The necrosis was sharply localized to this group of cells but appeared similar in all respects to that seen in the susceptible mice on the 5th day. Some cells were normal save for swollen, eosin-staining cytoplasm. The cytoplasm in others was eosin-staining and granular, or shrunken and surrounding nuclei in various stages of pycnosis. A few cells were entirely destroyed.

15 Days.—Virus was recovered from the two tested brains in the 10^{-1} dilution only. The remaining mice appeared well and one of three studied for brain lesions showed nothing abnormal. The other two showed perivascular accumulations of round cells throughout the brain, plus necrosis of pyramidal cells of olfactory bulbs and piriform areas, more extensive than in the 10 day mouse (Fig. 4). The cell exudate in the neighborhood was marked and there was present a glial cell proliferation not seen in sections of susceptible mice (Fig. 3).

20 Days.—No virus was recovered from the whole brains of the two tested mice but the two examined for brain lesions showed inflammatory changes in both superficial and deep tissue. Vessels were quite generally collared with round cells and both beneath the pia and deep in the brain, foci of round cells were scattered about the blood vessels.

Experiment 6.—Oct. 3, 1935. Thirty-one resistant and 118 susceptible mice were given an intranasal instillation of 0.03 cc. of virus diluted 1 to 100. 116 (98 per cent) of the susceptibles died of encephalitis within 9 days; four (12.9 per cent) of the resistants died on the 6th, 10th, 13th, and 25th days respectively.

Oct. 26, 1935. Twenty-two resistant and twenty-five susceptibles were given a similar intranasal instillation of virus resulting in the death from encephalitis of one (4.5 per cent) of the resistants on the 16th day and twenty-four (96 per cent) of the susceptibles by the 10th day.

The forty-eight resistant mice which survived these two tests were examined at intervals of 27 to 117 days after injection for brain and spleen lesions and also for the presence of active virus in the brain.

Virus was not recovered in the brains of any of these survivors tested 1 to 3 months after nasal instillation. Lesions, however, were marked on the first examination, 27 days, and were still present although resolving at 117 days.

At 27, 36 to 38, and 51 to 54 days, the seventeen mice examined showed a diminishing number of round cells collected beneath the pia and surrounding the

were sacrificed and tested for content of virus in various portions of the brain. At the same time, the nasal titre of the virus for susceptibles was checked. On the 7th day, three mice were sacrificed, their brains pooled, titred in susceptible mice both intracerebrally and intranasally, and passed again intranasally into eight more virus-resistant mice. Each day, one of these second passage, virus-resistant mice was sacrificed for tests on distribution of virus in brain and on the 7th day, three were sacrificed, brains pooled, titred intracerebrally and intra-

TABLE V

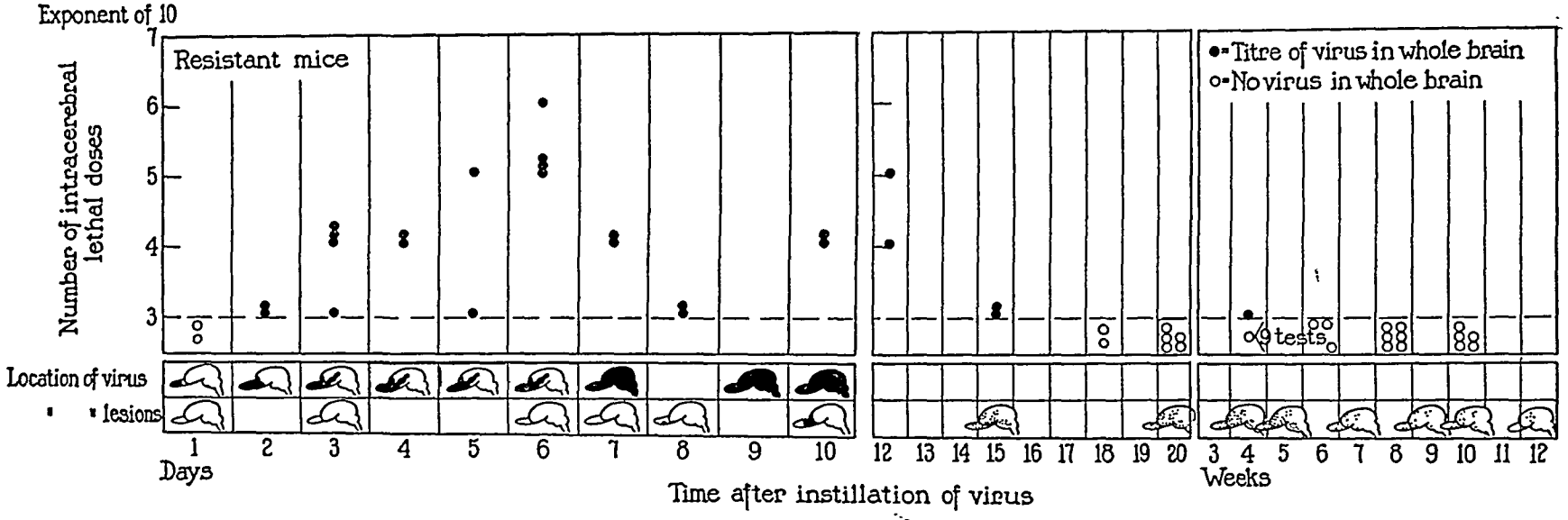
Alteration in Amount of St. Louis Virus after Nasal Passage through Resistant and Susceptible Mice

Source of virus Type of mouse	Virus titre tested in susceptible mice										
	Intracerebral titre 0.03 cc. to two mice							Nasal titre 0.03 cc. to two mice			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Susceptible	N.T.	N.T.	N.T.	N.T.	N.T.	4,* 5	5, 6	N.T.	N.T.	6, 6	6
Resistant	"	"	7, 7	12 (A)		N.T.	N.T.			N.T.	N.T.
1 passage											
Resistant	"	"				"	"		N.T.	"	"
2nd passage											
Resistant		"	N.T.	N.T.	N.T.	"	"		"	"	"
3rd passage											
Resistant 1 passage plus Susceptible 1 passage (A)	N.T.	"	"	"	"	6, 6	7, 8	7, 8	8, 8	8	"

* = duration of life of mouse in days.
N.T. = dilution not tested.
Blank spaces = mice remained well 21 days.
A = brain of susceptible mouse used for titration.

nasally as in the first passage, and finally passed for the third time intranasally into virus-resistant mice.

The virus carried as routine in brains of susceptible mice dropped 1,000-fold in amount after one intranasal passage in resistant mice. At the outset (Table V) it was fatal in 10^{-4} dilution intranasally and 10^{-7} dilution intracerebrally. This virus instilled into the noses of resistant mice (first passage) and recovered from their brains was not fatal for susceptible mice when given intranasally and was



TEXT-FIG. 2. Distribution of virus and lesions in resistant mice following nasal instillation of St. Louis virus.

the eleventh, fourteenth, and seventeenth passages. On each occasion, resistant brain virus injected into susceptibles intracerebrally was fatal not beyond the 10^{-4} or 10^{-5} dilution and intranasally was harmless.

In summary, virus in susceptible mice, when transferred to resistants, loses in quantity approximately 1,000-fold. This loss is such that after one passage in resistants the virus becomes non-infective

TABLE VI

Alteration in Amount of St. Louis Virus after Intracerebral Passage through Resistant and Susceptible Mice

Type of mouse	Brain titre of virus after intracerebral injection										
	Intracerebral titre 0.03 cc. to two or three mice							Nasal titre 0.03 cc. to three mice			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Susceptible	N.T.	N.T.	N.T.	N.T.	N.T.	4,* 5	5, 6	N.T.	N.T.	6, 6, 6	6, 8
Susceptible 1 passage	"	"	"	"	"	7, 7	7, 7	"	"	8, 8, 8	
Resistant 1 passage	"	"	"	8, 8, 8	8, 9, 9						
Resistant 1 passage plus Susceptible 1 passage (A)	"	"	"	5, 6	6, 6	6, 6	6	N.T.	8, 8, 9	8, 9, 9	9

* = duration of life of mouse in days.
N.T. = dilution not tested.
Blank spaces = mice remained well 21 days.
A = brain of susceptible mouse used for titration.

for susceptibles by the nasal route. If passed in series in resistants intracerebrally, the resistant brain virus remains active to the 10^{-4} dilution. Its nasal infectivity, however, is lost at once due probably to the fact that the required infecting dose, which is about 10,000 times the intracerebral infecting dose, is not contained in the resistant brain. Virus passed in resistants appeared to undergo no qualitative change and was restored to titre by one passage in susceptibles.

fatal to them by intracerebral route not beyond the 10^{-4} dilution. Again, this first passage virus in brains of resistant mice, when again instilled into the noses of resistant mice, second passage, failed to reach their brains and when these brains were pooled and tested, failed to kill susceptible mice either intranasally or intracerebrally. At the same time, when first passage virus in brains of resistants was placed in brains of susceptibles, and the brains of these susceptibles A, were titred after the animals had succumbed to encephalitis, the titre reverted at once to normal.

This experiment was repeated on three occasions with the uniform result that after one intranasal passage in resistants, the virus did not kill susceptibles when given intranasally in highest concentration and fell in titre 1,000-fold when injected intracerebrally in susceptibles. After a second intranasal passage in resistants, another 1,000 reduction in titre took place, rendering the second passage material generally non-infective for susceptibles.

These intranasal passage experiments were paralleled by intracerebral passages of virus in resistant mice.

Experiment 8.—Jan. 13, 1936. Virus was injected intracerebrally in 10^{-2} dilution into one susceptible and two resistant mice and at the same time was titrated intracerebrally and intranasally in susceptible mice. 6 days later, at a time when brain content of virus was presumably at a maximum, the animals were sacrificed, brains removed, pooled, and titrated intracerebrally and intranasally in susceptible mice. Finally, the brain of a susceptible mouse A, succumbing to the intracerebral injection of virus from the brain of a resistant mouse, was likewise titrated in susceptibles to determine whether one passage in susceptibles would restore the titre to maximum.

The results are given in Table VI. At the outset, the virus was fatal to susceptibles intracerebrally in 10^{-7} and intranasally in 10^{-4} dilutions. The brain of the test susceptible mouse receiving the virus showed a similar titre but that of the resistant mouse was fatal intracerebrally not beyond the 10^{-5} dilution and intranasally not at all. Finally, the brain of a susceptible A, which had succumbed to the 10^{-5} dilution of resistant brain virus given intracerebrally, was titrated in susceptibles and found to contain the usual amount of virus.

Serial intracerebral injections and passages in resistant mice were carried out twenty-two times and virus was tested for as above at

A further point previously stressed (1) in this connection is that the development and extension of lesions do not bear a simple relationship to the presence and quantity of virus. Virus given intranasally to resistant mice is demonstrable in the brain 7 days before lesions are recognized and disappears 2 months before lesions clear up. In fact, the foci and neighboring perivascular accumulations of leucocytes which are the outstanding and characteristic lesions of the disease in monkeys and man do not usually appear in the caudate nuclei until virus is no longer present. This dissociation of virus and lesions may well account for the failure to recover a specific agent from human cases in which lesions of this sort are conspicuous.

We have no definite conception of the essential difference between these resistant and susceptible mice save that the central nervous systems of the latter appear highly susceptible to a number of the encephalitis-producing viruses.

With regard to the St. Louis virus itself, the present experiments amplify those reported with susceptible mice (1), indicating that the virus is chiefly neurotropic, traveling from nose to brain by the olfactory route. Whether passage takes place by axons or perineural spaces is still uncertain, however, since lesions in resistant mice are an unreliable index of the presence of virus. The predominating picture is one of subpial and perivascular exudate of leucocytes and little or no conspicuous nerve cell involvement.

The 1,000-fold loss of titre of virus when passed from a susceptible to a resistant host and its prompt restoration when returned to a susceptible probably has a counterpart in less well controlled reactions between other viruses and hosts, such as the poliomyelitis virus and the monkey. Finally, in nature this salutary rôle of the resistant host in limiting multiplication of virus must be largely offset by the more dangerous one of acting as a reservoir of virus. Transfer of virus from a resistant to a susceptible with consequent increase in titre, together with reverse transfer from susceptible to resistant host with similar decrease may represent phenomena of special epidemiological significance.

CONCLUSIONS

1. St. Louis encephalitis virus injected intracerebrally or intraperitoneally in maximum doses in resistant mice is distributed and is

DISCUSSION

In discussing the implications of these findings, it must first be made clear that we are using two inbred strains of mice of identical parentage which differ widely and quantitatively in inborn resistance to the virus. At the same time, the individuals within each strain are mostly uniform in their response, indicating that variables not only genetic but also environmental have been controlled. A second point is that we deal here with resistance not alone to artificial infections following a parenteral injection of virus but to the more natural infections resulting from placing the virus on the external nares. And finally, we have sought to throw light on the resistance mechanism by analyzing extremely resistant and extremely susceptible individuals whose responses are generally predictable and applying the knowledge to the problem in average mice.

The experiments show that, *ceteris paribus*, inborn resistance factors determine the amount of mortality, type of clinical disease, persistence of virus, and type of pathology. Mice with inborn low resistance die promptly with fulminating encephalitis and show extensive destruction of the brain; mice with high resistance generally remain well and show microscopic focal lesions in the brain closely resembling those in the human disease. Finally, virus persists at least 3 weeks in the brains and spleens of resistant mice.[2]

The pathological findings suggest that there is, besides an etiological specificity of lesions, a host specificity. For example, the lesions in resistant mice following nasal instillation of St. Louis virus are extraordinarily like those in man. But they resemble also those in human cases of lethargic encephalitis, unnamed encephalitides, and certain types of bulbar and encephalo-poliomyelitis. Again, lesions in susceptible mice given St. Louis virus are entirely different from those in man but closely resemble those in mice following nasal instillation of louping ill and equine encephalomyelitis viruses, and are similar in general to those of yellow fever and vesicular stomatitis in mice. The character of a given lesion, therefore, is affected as much by host factors as by those of the specific agent.[3]

[2] A similar controlling effect of inborn resistance on the character of *B. enteritidis* mouse typhoid has been reported (3).

[3] Rake has separated quantitative differences in lesions in mice following nasal instillation of pneumococci dependent on inborn resistance factors (4).

PLATE 49

FIG. 3. 15 days. Piriform area similar to Fig. 2. Besides the subpial exudate, this unusual reaction was noted in two animals, consisting of round cells scattered more diffusely through the superficial layers, capillaries unusually conspicuous, a proliferation of glial cells, and scattered nerve cells undergoing necrosis. × 300.

FIG. 4. 15 days. Olfactory bulbs. Area similar to Fig. 1. The exterior band of pyramidal nerve cells is interrupted by an area, upper left in the figure, of necrosis. Nerve cells are absent altogether, or are shrunken, with deeply staining cytoplasm and pycnotic nuclei. Many nerve cells in the deeper molecular layers, lower left of figure, are also necrotic. This lesion was encountered in two mice. × 300.

PLATE 50

FIG. 5. 36 days. Caudate nuclei. This lesion was conspicuous in the greater percentage of mice examined between the 30th and 50th days. Mononuclear cells are collected in the perivascular spaces and in neighboring foci. These foci also contain an excess of glial cells and occasionally a necrotic nerve cell. × 300.

FIG. 6. 74 days. Caudate nuclei. Similar lesions showing two affected nerve cells in the midst of mononuclear leucocytes collected near a blood vessel (lower left of figure) which is itself surrounded by round cells. × 550.

effective in a manner generally similar to that in susceptible mice. The minimum infecting dose is at least 1,000 times greater in resistant than susceptible mice and virus injected in the brain tends to remain at a relatively low titre, persist for a few days, and then disappear.

2. Virus dropped in the nares is demonstrable and progresses in the brains of resistant mice as in susceptible mice, but does not increase in titre beyond the 5th day, does not bring about fatal encephalitis, and persists for at least 4 weeks.

3. Lesions in the brains of resistant mice following nasal instillation of virus do not appear until the 8th day, reach a maximum at 40 days, and are still present, though resolving, at 3 months. The changes resemble those seen in the human disease and in other unnamed forms of encephalitis.

4. The quantity of virus drops 1,000-fold when recovered from resistant mice and becomes non-infective by the nasal route. Passage in susceptible mice promptly restores its full titre.

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EXPLANATION OF PLATES

Sections of brains of resistant mice at various intervals after nasal instillation of St. Louis encephalitis virus. Eosin-methylene blue stain.

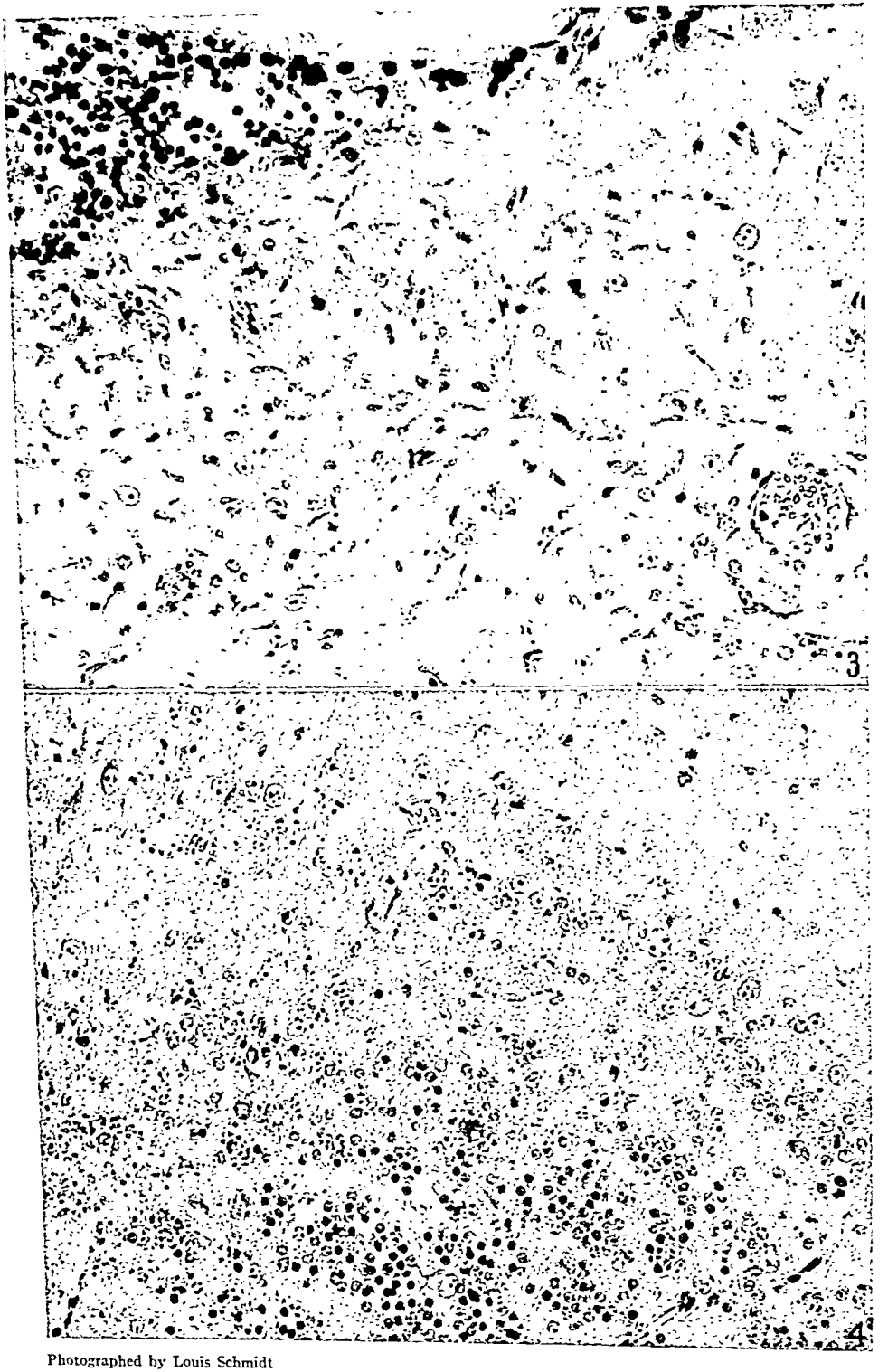
PLATE 48

FIG. 1. 8 days. Earliest lesion. Olfactory bulbs. Ventral, medial, posterior surface. One of several areas showing an exudate of round cells beneath the pia and extending between the olfactory nerve bundles toward the pyramidal cell layers. Nerve cells appear unaffected. This lesion is similar in every respect to that appearing in susceptible mice on the 3rd day (Reference 1, Fig. 1). × 500.

FIG. 2. 10 days. Early lesion. Piriform area. Ventral, medial surface. Round cells are collected in considerable numbers beneath the pia and in the Virchow-Robin spaces of neighboring blood vessels. Capillaries are prominent. Nerve cells appear normal. This lesion was present in most mice examined at this time and resembled precisely that noted in susceptible mice on the 4th day (Reference 1, Figs. 2, 3). × 300.

Photographed by Louis Schmidt

(Webster and Clow: Encephalitis in mice with high resistance)



Photographed by Louis Schmidt

(Webster and Clow: Encephalitis in mice with high resistance)

Photographed by Louis Schmidt

(Webster and Clow: Encephalitis in mice with high resistance)

PERSISTENCE OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS IN IMMUNE ANIMALS AND ITS RELATION TO IMMUNITY

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PLATE 51

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In a previous paper[1] it was reported that virus could be demonstrated in the blood and urine of mice and guinea pigs infected with acute lymphocytic choriomeningitis. Virus was also detected in nasal washings from mice infected naturally or experimentally. From an epidemiological viewpoint it is important to know whether or not the virus disappears from the blood, the secretions, and the excretions of immune animals.

Methods

The blood, urine, and nasal secretions of immune animals were tested for virus by inoculation into guinea pigs, which are highly susceptible and in which the disease can be diagnosed with certainty. Every infection, even the mildest subclinical one, produces in these animals a very solid immunity which can be detected by an inoculation with highly virulent virus given 3 to 4 weeks after the first inoculation. All guinea pigs which presented only fever or slight symptoms following inoculation were so tested.

Blood was repeatedly taken from the immune mice at long intervals in order not to disturb seriously the hematopoietic function. The blood was obtained by cardiac puncture under deep ether anesthesia, using a 0.25 cc. or 1 cc. tuberculin syringe and a very fine needle. At each bleeding 0.2 cc. blood was drawn and inoculated immediately (not defibrinated) into the brain of an etherized guinea pig. The heart puncture in mice proved to be a dangerous operation, but it was the only reliable method of obtaining standard amounts of blood. From guinea pigs blood was also obtained by cardiac puncture.

Urine was collected from mice and guinea pigs in sterile Petri dishes by exerting slight pressure on the abdomen in the region of the urinary bladder. Urine

[1] Traub, E., *J. Exp. Med.*, 1936, 63, 533.

toms. 3 weeks later serum was collected from them (about 0.2 cc. was obtained from each mouse), and the pooled serum was filtered through a Berkefeld N candle. The filtered serum produced the disease in a guinea pig (0.25 cc. inoculated intracerebrally). The virus in the serum was inactivated by heating at 56°C. for ½ hour and no antivirus was detected in the heated serum. In a preliminary experiment with immune guinea pig serum it had been found that such heating did not affect antivirus.

From thirteen other mice which were injected with virus by different routes and failed to show symptoms following the first inoculation and the intracerebral test inoculation, serum was collected 4 weeks after the latter test. The pooled serum was not filtered, and it produced the disease when injected intracerebrally into a guinea pig. The virus in this serum was also inactivated by heating and no antivirus was detected in the heated serum.

Experiments carried out to determine the duration of the infection in immune mice in relation to their immunity are recorded in Tables I, II, and III.

In the experiment given in Table I seven mice were used which were left over from different experiments and which had received their first inoculations on different days. Mice 1 to 5 were obtained from the infected colony. Mice 3 and 4 had probably undergone a previous natural infection, since neither showed symptoms following an intracerebral inoculation with 0.04 cc. of virulent 5 per cent mouse brain suspension. Mice 1 and 2, which showed typical symptoms following intracerebral inoculation with the same amount of virus and recovered, had probably not been infected prior to the inoculation. Mouse 5 may have been infected prior to the intranasal instillation of 0.05 cc. of the supernatant fluid of a 5 per cent mouse brain suspension, because intranasal instillations of virus in mice as a rule produce no symptoms. Mice 6 and 7 were descended from the infected stock, but were bred from disease-free parents and were not infected prior to the intranasal instillation of a 1 per cent guinea pig brain suspension carried out according to the method described previously.[1] The results of the blood tests are given in Table I.

Table II records tests for virus in seven mice which were inoculated intraperitoneally with 0.5 cc. of a 1 per cent guinea pig brain suspension, were very sick from the 7th to about the 14th day after the inoculation, and recovered. On the 27th day their immunity was tested by intracerebral inoculation with virus. The surviving mice were again inoculated intracerebrally 83 and 166 days, respectively, after the first test for immunity and none of them showed symptoms.

obtained in this manner was always free from blood but contaminated with bacteria, which, however, never interfered with the outcome of the experiments. Each urine sample was injected subcutaneously into the planta of a guinea pig.

Nasal washings were taken on mice and guinea pigs by repeatedly immersing their nostrils, each time for 5 to 10 seconds, in physiological saline (0.5 to 1 cc. per mouse) contained in a sterile Petri dish. The animals were not anesthetized. The nasal washings appeared slightly turbid. The saliva was not eliminated with certainty by this procedure. The inoculations were made subcutaneously into the plantae of guinea pigs. Bacteria present in the nasal secretions proved to be harmless to the test animals.

Mice were tested for immunity by intracerebral inoculation, under ether anesthesia, with 0.04 cc. of an infectious 5 per cent suspension of mouse brain. A group of 5 to 8 normal control mice from a stock free from choriomeningitis was included in each test. All control mice died in typical convulsions 6 to 7 days after the inoculation, while the mice whose immunity was being tested showed no symptoms, as indicated in Tables I, II, and III. Immunity tests in guinea pigs were carried out by a subcutaneous inoculation (0.25 cc. into each planta) with a virulent 10 per cent suspension of guinea pig brain. At least two normal control animals were inoculated.

As a source of virus for neutralization tests a 10 per cent suspension in saline of the brain of a guinea pig which had been killed when moribund following subcutaneous inoculation with a very virulent strain of the virus was used. For the tests this suspension was centrifugalized and known dilutions of the supernatant were mixed with equal amounts of undiluted serum. After incubation for ½ hour at room temperature, 0.25 cc. of each mixture was inoculated subcutaneously into each planta of a guinea pig. A comparative test has shown that smaller amounts of antivirus can be detected by the subcutaneous inoculation of guinea pigs than by the intracerebral injection of mice. The former were therefore used in these tests.

Tests for Virus in Blood, Urine, and Nasal Washings from Immune White Mice

The first observations on the persistence of active virus in the blood of immune mice were made with groups of mice from the infected stock which were inoculated with infective mouse brain suspension by different routes (15 mice intranasally, intraperitoneally, intracutaneously, and intracerebrally, respectively). Ten intracerebrally injected mice died, while the other 50 mice showed no symptoms. These mice were tested for immunity by an intracerebral inoculation with 0.04 cc. of an infective 1 per cent mouse brain suspension 3 weeks after the first inoculation and none of them showed any symp-

TABLE II

Tests for Virus in the Blood and Urine of Immunized Mice

Time after 1st test of immunity	Inoculations into guinea pigs of heart blood from							Inoculations into guinea pigs of pooled urine from mice 8–14	
	Mouse 8	Mouse 9	Mouse 10	Mouse 11	Mouse 12	Mouse 13	Mouse 14	Amount	Result
days								*cc.*	
30	+ F, S, I	0	0	+ F, S, I	+ D 27	0	+ D 21	0.7	+ F, S, I
65	Died of injury on	0	0	" " "	+ D 15	0	+ D 17	0.25	" " "
83*	30th day	0	0	" " "	+ D 17	0	Died of injury on	—	—
98		+ F, S, I	+ F, S, I	" " "	+ F, severe S, I	+ F, S, I	65th day	1.4	0
133		0	0	0	+ F, S, I	0		0.85	0
166*		0	0	—	—	—		—	—
175		Killed on 166th day. No virus detected in organs		Blood plasma + D17 Washed blood cells 0	Blood plasma + F, severe S Washed blood cells 0	Blood plasma + D 17 Washed blood cells 0		—	—
207				0	+ F, S	0		—	—

+ = virus detected. 0 = no fever, no illness, and no immunity. D 27 = died in 27 days. — = no test made. F = fever. S = typical symptoms. I = immunized.

* The surviving mice were tested for immunity.

TABLE I

Tests for Virus in the Blood of Immunized Mice

Mouse No.....	1	2	3	4	5	6	7
Time after 1st inoculation	1st inoculation ic followed by typical symptoms and recovery Test inoculation 50 days after 1st inoculation	Inoculated ic Typical symptoms, recovery No test inoculation	Inoculated ic. No symptoms No test inoculation	1st inoculation ic No symptoms Test inoculation ic 28 days after 1st inoculation	1st inoculation inas. No symptoms Test inoculation ic 28 days after 1st inoculation	Test inoculation ic 15 days after 1st inoculation	1st test inoculation ic 15 days after 1st inoculation 2nd test inoculation ic 143 days after 1st inoculation
days	Results of blood inoculations (0.2 cc. heart blood of each mouse injected ic into a guinea pig)						
Shortly before 1st inoculation	—	—	—	—	—	0	0
11	—	—	—	—	—	D 16	D 15
26	0	F, S, I Died of injury following bleeding on 26th day	D 20	—	—	—	—
28	—		—	—	0*	—	—
42	—		—	0*	0	—	—
50	0*		D 16 Died of injury following bleeding on 50th day	0 Died of injury following bleeding on 42nd day	Killed for serum on 70th day	0	D 14
64	0					—	D 25
110	Killed for serum on 92nd day after 1st inoculation					—	0
135						0	0
143						0	0*
158						Died of injury following bleeding on 143rd day	F, S, I
178							0

ic = intracerebrally; inas = intranasally. 0 = no fever, no symptoms, not immunized. D 16 = died in 16 days. — = no test made. F = fever. S = typical symptoms followed by recovery. I = immunized.

* The test inoculations were made immediately after the bleedings.

TABLE III

Tests for Virus in the Blood, Urine, and Nasal Washings from Mouse 15

Time after removal from infected stock	Blood	Urine		Nasal washings	
	Result (0.2 cc. injected ic into 1 guinea pig)	Amount	Result (each specimen injected sc into 1 guinea pig)	Amount	Result (each specimen injected sc into 1 guinea pig)
hrs.		*cc.*		*cc.*	
1	D 15				
days					
25	F, no S, I				
49	F, slight S, I				
64	D 17				
110	F, slight S, I	0.3	F, no S, I		
123		0.35	F, severe S, I		
126		0.2	F, severe S*		
127		0.1	" " "		
135		0.15	" " "	0.7	F, severe S*
143†	F, no S, I				
158	F, severe S*	0.02	D 12		
162				0.7	D 16
168		0.02	D 15	0.7	F, slight S, I
184		0.01	F, slight S, I	0.7	F, severe S*
192		0.15	F, severe S, I	0.8	F, severe S, I
198		0.05	F, no S, I	0.8	" " " "
213		0.4	F, severe S, I		
214		0.02	" " " "		
215		0.02	" " " "		
216		0.01	" " " "		
225	Blood plasma and washed blood cells F, severe S*				

D 15 = died in 15 days.

F = fever.

S = typical symptoms of choriomeningitis followed by recovery.

I = immunized.

* Since the guinea pig showed unmistakable symptoms, no test inoculation was made.

† Tested for immunity by intracerebral inoculation with virus immediately after bleeding.

the disease in guinea pigs. No attempt was made to determine how many mice had remained carriers of the virus.

The experiments just presented indicate that some mice remain carriers of the virus for several months after clinical recovery, while

Blood and organs from mice 9 and 10, killed on the 166th day, were examined. No virus was detected in the brain and spinal cord, lungs, heart, liver, spleen, kidneys, urinary bladder, salivary glands, uteri, ovaries, or several lymph nodes including the mediastinal and mesenteric ones. These determinations were made by injecting the total amount of each suspension of an entire organ into a guinea pig (amounts of 2 cc. and less were injected subcutaneously into the plantae; if there was more suspension than 2 cc., the remainder was injected intraperitoneally). The suspensions of several small organs, for instance those of the salivary glands and submaxillary lymph nodes or of the ovaries and the uteri, were pooled and so injected.

On the 175th day the blood plasma and washed blood cells of mice 11, 12, and 13 were tested for virus. 0.2 cc. heparinized plasma from each mouse was injected intracerebrally into a guinea pig. The blood cells from each mouse were washed three times in 10 cc. Tyrode solution containing heparin. From the centrifuged washed blood cells of each mouse the upper layer containing the bulk of the white cells and many erythrocytes was drawn off with a capillary pipette, and enough Tyrode solution was added to make each inoculum 0.25 cc., which was injected intracerebrally into a guinea pig. Virus was detected in the heparinized plasma of all three mice, but not in the washed blood cells.

Table III records tests for virus made upon mouse 15 over a period of more than 7 months. This animal, which had undergone a natural infection, was about 7 weeks old when it was removed from the infected stock. It grew normally and appeared to be in perfect health throughout the experiment. Its immunity was confirmed by intracerebral inoculation 143 days after its removal from the colony. The regularity with which virus was detected in the urine and nasal washings is remarkable. The virus seems to have been continuously present in the urine, and very small amounts of urine produced the disease in guinea pigs.

On the 225th day mouse 15 was sacrificed for histological examination and virus was demonstrated in the blood plasma and washed blood cells as well as in the thoroughly washed spleen tissue. Other organs were not tested for virus.

In another experiment the nasal washings of eleven immune mice were tested for virus. These mice had been infected by intravenous injection with 0.25 cc. supernatant fluid of a virulent 10 per cent guinea pig brain suspension. Six mice were sick from the 6th to the 10th day after the inoculation and then recovered completely, while five showed no definite symptoms. Pooled nasal washings obtained on the 39th, the 56th, and the 121st day after inoculation produced

ing 0.2 cc. intracerebrally into a guinea pig, with a negative result. The symptoms in the two guinea pigs whose urine contained virus had been severe, while in the others they were mild.

In another experiment virus was detected in pooled urine from five guinea pigs that had shown severe symptoms following subcutaneous inoculation with virus 35, 36, 37, 40, or 44 days previously. The animals received no tests for immunity. Shortly after the collection of the urine each guinea pig was bled, and its defibrinated blood was injected into a guinea pig (0.2 cc. intracerebrally, 3 cc. intraperitoneally), with negative result. Pooled nasal washings taken from these five guinea pigs were avirulent. No other tests have been made with nasal washings from immune guinea pigs.

Virus was also present on the 23rd day after inoculation in the urine of a guinea pig which had recovered from choriomeningitis of moderate severity. The blood serum of this guinea pig was avirulent and contained some antivirus at that time (test recorded in Table V).

Experiments Bearing on the Mechanism of the Immunity to Choriomeningitis

The immunity produced in mice by inoculation with virus arises rapidly. When mice injected intraperitoneally with virus are given an intracerebral test inoculation on the following day, they all die in typical convulsions. When, however, the test inoculation is given 5 days after the first injection, some mice will be immune. Virus injected intracerebrally on the 8th day has no effect, even though some mice still show symptoms of the disease. The occasional persistence of virus in the blood of mice after recovery suggested an investigation into the relation between the presence of the virus and immunity.

Mice in whose blood no virus had been demonstrated and others whose blood was virulent were tested for immunity by intracerebral injection with virus. Records on such mice are given in Tables I, II, and III (mice 1, 4, 5, 7, 9, 10, 11, 12, 13, and 15). It is evident that mice which carried virus in their blood were just as resistant to reinoculation as those which did not. Numerous other tests carried out with mice from the infected stock confirmed this observation.

It was of interest to determine whether circulating antivirus ac-

others cannot be shown to carry it after a short time. The number of mice examined is too small to allow a conclusion on the percentage of immune carriers.

Tests for Virus in Blood, Urine, and Nasal Washings from Immune Guinea Pigs

Tests have been made with blood of guinea pigs (whole blood, washed corpuscles, and plasma were tested in two cases) drawn on

TABLE IV

Tests for Virus in the Urine of Immune Guinea Pigs

Time after test of immunity	Guinea pig 1		Guinea pig 2		Guinea pig 3		Guinea pig 4		Guinea pig 5	
	Character of disease									
	Very severe (31 days)*		Very severe (31 days)*		Fever, no symptoms (37 days)*		Fever, slight symptoms (30 days)*		Fever, no symptoms (30 days)*	
	Subcutaneous inoculations of urine into guinea pigs									
	Amount	Result	Amount	Result	Amount	Result	Amount	Result	Amount	Result
days	*cc.*		*cc.*		*cc.*		*cc.*		*cc.*	
18	0.15	+ D 17	0.25	+ D 13	0.8	0	0.5	0	0.7	0
19			0.5	+ D 22						
27										
35	0.5	0	0.2	0						

\+ = virus detected.
0 = no fever, no symptoms, and no immunity.
D 17 = died in 17 days.
* Number of days which elapsed between inoculation and test of immunity.

the 35th day after the immunizing inoculation or later, but we have thus far been unable to demonstrate virus in it, even when several cubic centimeters were injected into susceptible guinea pigs. In the sera of immune guinea pigs antivirus is readily demonstrable and is probably responsible for the inactivation of the virus in the blood.

Tests for virus in the urine of five immune guinea pigs are recorded in Table IV. These animals were infected by subcutaneous inoculation with virus and their immunity was tested in the same way. The blood of each guinea pig was tested for virus by inoculat-

Histological Examination of Tissues from Immune Animals Which Carried Virus

Material from seven naturally infected mice was examined histologically. These mice had shown no clinical evidence of disease while under observation and carried virus in their blood for several months. Virus was demonstrated in the blood of each mouse shortly before it was chloroformed.

Mouse 15 (Table III), killed on the 225th day after its removal from the infected stock, showed at autopsy a somewhat enlarged spleen and a nutmeg colored liver. No other changes were noted. The lungs appeared normal. The brain, spinal cord, lungs, heart, liver, spleen, kidneys, urinary bladder, as well as the femoral and vertebral bone marrow, were examined histologically. The lungs showed areas of marked interstitial pneumonia. There was an interstitial hepatitis and large round cell collections in the neighborhood of blood vessels (Fig. 1). Smaller collections of lymphocytes and some polymorphonuclear leucocytes were scattered over the section and gave it a spotted appearance. Kidney sections showed small areas of interstitial nephritis. In the spleen the Malpighian bodies were enlarged, and the number of megakaryocytes was increased. In the rest of the material studied no definite changes were noted.

Mouse A carried virus in the blood for at least 3 months. At autopsy no definite changes were seen. The liver, spleen, and kidneys were examined histologically. In the liver there was a patchy hyperplasia of Kupffer cells. The spleen showed no lesions, but sections through the kidneys presented small areas of interstitial nephritis.

Mouse B carried virus in the blood for at least 5 months. No changes were found at autopsy. Histologically a reticuloendothelial hyperplasia was detected in the liver, and kidney sections showed a patchy interstitial nephritis. The spleen appeared normal. No other organs were examined.

Mouse C carried virus in the blood for at least 4 months. At autopsy no definite changes were noted. The lung section showed areas of interstitial pneumonia. In the liver and spleen no definite changes were present. The kidneys showed a slight interstitial nephritis.

Mouse D carried virus in the blood for at least 6 months. Its respiratory rate was increased. At autopsy a mediastinal tumor was noted which filled about one-half of the thoracic cavity. The spleen was enlarged (about two to three times normal volume). Histologically the tumor appeared as a lymphosarcomatous mass containing an extremely large number of cells in mitosis. The lungs presented an interstitial pneumonia with round cell collections in the neighborhood of blood vessels and bronchi and in the lung tissue. The liver showed small round cell collections around blood vessels and bile ducts. In the spleen the Malpighian

counted for the apparent disappearance of the virus from the blood of some immune mice. Neutralization tests were therefore carried out with three sera obtained from immune mice in whose blood no virus was detected in repeated tests.

Immune Serum I.—Pooled sera from mice 1 and 5 (Table I). Subcutaneous injection of 0.25 cc. of this serum into a guinea pig produced no disease.

Immune Serum II.—Pooled sera from mice 9 and 10 (Table II) drawn on the 166th day, and from mouse 7 (Table I) drawn on the 227th day. Intracerebral inoculation of 0.3 cc. of this serum into a guinea pig was without effect.

Immune Serum III.—Pooled sera from five immune, full grown mice from the infected stock. A guinea pig inoculated intracerebrally with 0.3 cc. of this serum showed no reaction.

TABLE V

Neutralization Tests with Three Sera from Immune Mice and the Serum of a Convalescent Guinea Pig Whose Urine Contained Virus

Virus dilution	Results of inoculations of the serum-virus mixtures subcutaneously into guinea pigs (1 guinea pig used for each mixture)						
	Experiment 1			Experiment 2			
	Immune mouse serum I	Normal mouse serum (control)	Normal horse serum (control)	Immune mouse serum II	Immune mouse serum III	Normal mouse serum (control)	Convalescent guinea pig serum
10^{-1}	+	+	−	−	−	−	−
10^{-2}	+	+	+	+	+	+	+*
10^{-3}	+	+	+	+	+	+	+*
10^{-4}	0	+*	0	+	+	+	0
10^{-5}	−	0	0	0	0	0	−

\+ = died of choriomeningitis or was killed when very sick.
0 = showed no fever or symptoms.
− = not tested.
* Delayed disease.

The tests presented in Table V show that all three of these sera had practically no neutralizing power under the conditions of the experiment.

In contrast to the results in mice, numerous tests with sera from immune guinea pigs have invariably shown antivirus to be present.

Dr. T. F. McNair Scott of New York has kindly permitted me to refer here to his recent experiments in which he detected both lesions and virus in the central nervous systems of mice which had recovered from choriomeningitis following intracerebral inoculation with virus administered 14 or 28 days previously.

DISCUSSION

From the histological findings just outlined it seems possible that in animals which have recovered clinically from choriomeningitis but continue to carry active virus and to discharge it, the virus persists in lesions in the lungs, kidneys, and liver. From these lesions it may get into the circulation, and from the blood into the urine, or directly into the urine if lesions are in the urinary system. The lung lesions in mouse 15 were perhaps responsible for the presence of virus in the nasal secretions. The virus seemed to have multiplied continuously in this animal, because it discharged considerable amounts of virus over a long period of time. The presence of slight lesions in mice which once were infected, but in whose blood no virus was detected, may be taken to indicate that the discharging of virus into the blood ceases before the lesions have completely disappeared. In guinea pigs immune to choriomeningitis, in whose blood antivirus is present but which discharge virus with their urine, the virus may be situated intracellularly in kidney lesions and thus protected from the action of circulating antivirus.

In mice 6, 7, and 11, as well as in several other naturally infected mice not dealt with here, the virus gradually disappeared from the blood in the course of time. Neutralization tests indicated that circulating antivirus was not responsible for this gradual elimination, which perhaps ran parallel with the healing of the lesions.

When immune mice whose blood has been avirulent on repeated tests are reinoculated, the virus may circulate for some time (mice 7, 9, 10, 11, and 13; in mice 1, 4, and 5 the virus probably also circulated for some time following the test inoculation but was eliminated more rapidly than in the former mice). It is unlikely that new lesions are produced by the reinoculations. The time of circulation of the virus is comparable with that of antivirus in passively immun-

bodies were enlarged, and the red pulp was infiltrated with lymphocytes. Kidney sections showed a slight interstitial nephritis.

Mouse E carried virus in the blood for at least 6 months. At autopsy no gross lesions were noted. Histologically an interstitial pneumonia (Fig. 2) and a marked interstitial nephritis (Fig. 3) were detected. The liver, spleen, and mediastinal lymph nodes showed no definite changes.

Mouse F carried virus in the blood for at least 6 months. At autopsy no gross lesions were present. A slight interstitial pneumonia and a marked interstitial nephritis were noted in sections. The liver tissue contained small collections of round cells. The spleen and mediastinal lymph nodes appeared normal.

In the mice examined lesions were most frequently found in the lungs and kidneys. While it cannot be proved that these lesions are caused by choriomeningitis virus, it must be noted that they resemble the more marked lesions which are present in mice acutely ill following intravenous injection with the virus.

Sections through the lungs, liver, spleen, and kidneys of two full grown mice from the infected stock which had themselves been infected with choriomeningitis, but in whose blood no virus was detectable, showed no changes. In four other mice slight changes of the same character as those described above, but less marked, were noted. Traces of an interstitial pneumonia and small round cell collections near blood vessels were present in one mouse. In three cases small interstitial round cell collections were seen in liver sections. They were much less extensive than those in mice carrying virus. In one mouse a marked, patchy interstitial nephritis was noted, while in three cases only traces of interstitial nephritis were found.

Tissues from seven normal mice were examined histologically. These mice were bred from disease-free parents, and tests made shortly before the tissues were removed disclosed no pathogenic virus in the blood. The lungs and kidneys showed no lesions. In the lungs there were no peribronchial, perivascular, or interstitial round cell collections, such as are believed by some investigators to be present under normal conditions. Liver sections showed no changes in five cases, while in two cases a few small collections of polymorphonuclear leucocytes and round cells were present in the interstitial tissue. The collections consisted of less than ten cells each and were much less numerous and extensive than those noted in mice carrying the virus. There was no hyperplasia of Kupffer cells.

A guinea pig which had recovered from choriomeningitis of moderate severity was sacrificed on the 28th day after the subcutaneous inoculation with virus. It discharged virus with the urine at that time, but no virus was detected in the blood. Kidney sections revealed large perivascular round cell infiltrations and a marked interstitial nephritis very similar to that noted in mice.

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bodies were enlarged, and the red pulp was infiltrated with lymphocytes. Kidney sections showed a slight interstitial nephritis.

Mouse E carried virus in the blood for at least 6 months. At autopsy no gross lesions were noted. Histologically an interstitial pneumonia (Fig. 2) and a marked interstitial nephritis (Fig. 3) were detected. The liver, spleen, and mediastinal lymph nodes showed no definite changes.

Mouse F carried virus in the blood for at least 6 months. At autopsy no gross lesions were present. A slight interstitial pneumonia and a marked interstitial nephritis were noted in sections. The liver tissue contained small collections of round cells. The spleen and mediastinal lymph nodes appeared normal.

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Tissues from seven normal mice were examined histologically. These mice were bred from disease-free parents, and tests made shortly before the tissues were removed disclosed no pathogenic virus in the blood. The lungs and kidneys showed no lesions. In the lungs there were no peribronchial, perivascular, or interstitial round cell collections, such as are believed by some investigators to be present under normal conditions. Liver sections showed no changes in five cases, while in two cases a few small collections of polymorphonuclear leucocytes and round cells were present in the interstitial tissue. The collections consisted of less than ten cells each and were much less numerous and extensive than those noted in mice carrying the virus. There was no hyperplasia of Kupffer cells.

A guinea pig which had recovered from choriomeningitis of moderate severity was sacrificed on the 28th day after the subcutaneous inoculation with virus. It discharged virus with the urine at that time, but no virus was detected in the blood. Kidney sections revealed large perivascular round cell infiltrations and a marked interstitial nephritis very similar to that noted in mice.

of lymphocytic choriomeningitis contained virus for a few weeks after recovery, while that from mild cases contained no virus. Virus was never demonstrated in the blood of immune guinea pigs. Anti-virus was readily detected in it.

EXPLANATION OF PLATE 51

FIG. 1. Section through liver of mouse 15. Large round cell collection. Note area of interstitial hepatitis. Hematoxylin and eosin. × 220.

FIG. 2. Section through the lung of immune mouse E showing area of slight interstitial pneumonia with round cell collections. Hematoxylin and eosin. × 220.

FIG. 3. Section through kidney of immune mouse E showing an area of marked interstitial nephritis. Hematoxylin and eosin. × 332.

ized animals. In guinea pigs passively immunized to pseudorabies this time was found to vary between 1 and 4 weeks (unpublished experiments).

The immunity to choriomeningitis in mice does not seem to be dependent upon the presence of active virus in the body (mice 9 and 10). Unfortunately it cannot be definitely proved with the methods available at present that the whole body of the animal is free from virus, since subinfective amounts of virus may not be recognized. There is, however, reason to believe that the process of gradual elimination of the virus mentioned above continues to completion. Most of the evidence obtained with other viruses is in favor of this assumption. The evidence for the contrary view is not convincing.

While antivirus may be present in too low a concentration to be detected by the method employed, the failure to demonstrate it in sera from solidly immune mice is evidence that it does not determine the immunity of mice to choriomeningitis. The continued presence of virus in the blood plasma of immune mice (mice 11, 12, and 13) suggests that phagocytes also play no part in the immunity. It is tentatively concluded that tissue immunity is the essential factor in mice. In immune guinea pigs whose sera invariably possess neutralizing properties, the antivirus may be an essential immunity factor.

SUMMARY

In some apparently healthy mice the virus of lymphocytic choriomeningitis persisted for a considerable period of time after recovery, in the blood, urine, and nasal secretions, while in other mice it soon became undemonstrable. It is possible that the persistence of the virus is due to lesions in the lungs, liver, and kidneys.

The immunity to lymphocytic choriomeningitis in mice does not seem to depend upon the presence of virus in the blood and the organs tested. No antivirus was detected in sera from several solidly immune mice, which fact suggests that circulating antivirus plays no important part in their immunity. Leucocytes also seem to be no essential factor in this immunity, which probably is closely linked with the tissues.

The urine of guinea pigs which had recovered from severe attacks

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THE PROTECTIVE ACTION OF NASALLY INSTILLED IMMUNE SERUM AGAINST INFECTION WITH CERTAIN NEUROTROPIC VIRUSES BY WAY OF THE NOSE*

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The systemic administration of antiviral serum has been shown to be beneficial as a prophylactic measure in certain virus diseases, notably measles. It has proved difficult, however, to establish experimentally a general passive immunity against others, particularly in larger hosts and against viruses which invade the central nervous system by way of peripheral neural routes (1, 2). Protection of selected skin sites by the local injection of immune serum prior to the inoculation of certain dermotropic viruses has been demonstrated by several investigators (3–5). This local protection or passive immunity of the skin persisted for several days and in the case of pantropic, neuroinvasive viruses, such as pseudorabies and B virus, it was found that not only was the skin lesion prevented but also invasion of the central nervous system (6).

Since the nasal route is considered to be the portal of entry for many virus infections, it was deemed advisable to investigate the possibilities and limitations of local prophylaxis by means of specific antiviral serum. If the method of local protection in the skin were followed, it would be necessary to infiltrate the entire nasal mucosa with immune serum, which clearly is not practical. It has been observed, however, that minced susceptible tissue upon contact with antiviral serum was capable of fixing protective substance to such an extent that upon removal of the tissue from the serum and even after one or two washings it would remain refractory to infection with virus in *in vitro* cultures (5). It appeared possible, therefore, that

* Work begun at the Lister Institute, London, during the tenure of a National Research Council Fellowship in Medicine for 1934, and completed at The Rockefeller Institute.

centrifuged suspension of rabbit brain virus was instilled in each nostril. All of the guinea pigs treated with normal swine serum died, while an occasional one of those treated with immune serum survived. It appeared, however, that better results might perhaps be obtained by increasing the amount of immune serum to be instilled and diminishing the volume of the infective dose to a minimum in order to prevent it from getting into the lungs and to approximate natural conditions of infection by this route. The serum was increased to 0.5 cc. per nostril, administered slowly from a blunt, smooth edged, glass pipette, and the virus was reduced to 0.05 cc. for each nostril—an amount still larger than that ordinarily transmitted by natural droplet infection. A dose of 0.05 cc. per nostril of a 0.1 per cent virus suspension killed about half the number of control guinea pigs, while with the same amount of a 1 or 2 per cent suspension, only an occasional one survived. In view of variations encountered with different virus preparations, each experiment necessarily had its own controls, and 1 per cent virus suspension was used when made with fresh brain and 2 per cent or 5 per cent with glycerolated tissue.

Effect of Intranasal and Intramuscular Administration of Anti-pseudorabies Serum.—The purpose of this experiment was (*a*) to determine whether the nasal instillation of immune serum would protect against a small, yet ordinarily fatal, amount of pseudorabies virus introduced in the nose 2 hours later; (*b*) to determine whether an immune serum homologous for the test animal, *i.e.*, guinea pig in this case, might be more effective than the heterologous swine serum, and (*c*) to determine, in part at least, whether the effect of the nasally instilled serum is local, or remote after absorption, by comparing it with that produced by the intramuscular injection of an even larger amount of immune serum.

Sixteen guinea pigs were divided into four groups. One was untreated; the animals of the second group each received 3 cc. of swine antipseudorabies serum intramuscularly, while those of the third group were given 0.5 cc. of the same serum in each nostril; the guinea pigs of the fourth group received 0.5 cc. in each nostril of guinea pig antipseudorabies serum.[2] 2 hours later all animals were instilled in each nostril with 0.05 cc. of a 2 per cent virus suspension.

The results are shown in Table I. All the guinea pigs which received the immune serum intranasally survived without showing any signs of disease, while all those which were given the serum intramuscularly and three of the four untreated ones died with typical

[2] Prepared and kindly supplied by Dr. R. E. Shope.

the cells of the nasal mucosa might respond in a similar manner when flushed with antiviral serum.

EXPERIMENTAL

The virus of pseudorabies (Aujeszky's disease, infectious bulbar paralysis, mad itch) was selected for most of the experiments because its properties rendered it particularly suitable for this type of investigation. It produces a rapidly fatal (2 to 4 days) encephalitis in guinea pigs, following a single nasal instillation of minute amounts of virus. This virus invades the blood stream and viscera as well as the central nervous system and, as shown by Hurst (*7a*, *7b*), in rabbits it reaches the central nervous system by way of the peripheral nerves. As a result of its almost constant and rapid destructive effect on the sensory ganglion cells which are first attacked, the sites, which the infected animals scratch or bite, serve to indicate the nerves along which the virus, in all probability, reached the central nervous system from the periphery (*7a*). Guinea pigs infected intranasally scratch along the nose, the eyes, the ears, or the jaw. This "marking or indicating" property of the virus appeared particularly useful for these experiments, since it would tend to show whether in treated animals encephalitis was induced by way of the nerves from the nose, or whether the virus had gotten through the possibly protected region of the nose into the blood stream and reached the central nervous system by way of the nerves supplying the viscera.

It has already been shown that while pseudorabies antiviral serum mixed with virus *in vitro* has no protective effect even against minimal doses when injected intracerebrally in guinea pigs, it does prevent infection when administered by the nasal route (6). It remained to be determined, therefore, whether immune serum instilled in the nose prior to the administration of virus by this route would exert a protective effect.

The pseudorabies virus (Aujeszky strain) was always used in the form of fresh or glycerolated rabbit brain, obtained after intracerebral injection of the virus. Swine antipseudorabies serum[1] was used in most of the experiments. In preliminary tests 0.25 cc. of antiviral serum or of normal swine serum was dropped into each nostril, and an hour or two later, 0.25 cc. of a 10 per cent

[1] Kindly supplied by Dr. R. E. Shope.

from the nose than from muscle is not significant, because immune serum, even when mixed with pseudorabies virus *in vitro*, appears to be incapable of preventing infection when the mixture is injected intracerebrally in guinea pigs (6). In view of the fact that pseudorabies can spread in the body by routes other than the neural, it is interesting to observe that protection of the "first line" of cells to be attacked apparently also acts as a barrier to the further dissemination of the virus to other parts of the body where the cells are fully susceptible.

It may be said by some that the virus is "neutralized" by the immune serum, which obviously must remain adherent to the nasal mucosa, and for that reason is inactive wherever it may spread. But previous experiments on the mechanism of the neutralizing or protective action of antipseudorabies serum (5, 8) which showed no evidence of union between the virus and the protective antibody during the consummation of the immune process, make it difficult to accept this explanation. The experiments just referred to (5) also indicated that susceptible cells rendered refractory in the presence of immune serum are capable of fixing virus apparently to the same extent as normal cells, but that the virus fixed to such refractory cells fails to multiply and becomes inactive at a rate which varies with different viruses at body temperature under conditions unfavorable to multiplication. On this basis, one can imagine how a superficial layer of protected cells may act as a barrier to deeper dissemination of the virus, provided the dose is not too large, without resorting to a concept of direct "neutralization."

Duration of Protective Action of Immune Serum in the Nose and Effect of Certain Chemical Substances.—

To determine the duration of the protective effect of nasally instilled immune serum, three groups of four guinea pigs were treated with 0.5 cc. of swine antipseudorabies serum in each nostril. One group received the serum 24 hours before, another 5 hours before, and the third, 2 hours before the time when they were all given 0.05 cc. of a 2 per cent virus suspension in each nostril. Four guinea pigs were treated with 1 per cent tannic acid (0.5 cc. in each nostril) to determine the effect of a single treatment with an astringent, protein-precipitating substance (9). 0.1 per cent neutral acriflavine was used in a similar manner in another four guinea pigs; this chemical has been reported by Craigie and Tulloch (10) as capable of rendering the rabbit skin refractory to

signs of pseudorabies on the 3rd day after inoculation. This experiment indicates that (*a*) immune serum, either homologous or heterologous as regards the host, instilled into the nose, is capable of protecting the host for a period of at least 2 hours against invasion with

TABLE I

Comparative Effect of Intranasal and Intramuscular Administration of Antipseudorabies Serum on Susceptibility of Guinea Pigs to Infection by the Nasal Route

Type of antipseudorabies serum*	Amount and route	Guinea pig No.	Result
Guinea pig	0.5 cc. in each nostril	1	S†
		2	S
		3	S
Swine	" " " " "	4	S
		5	S
		6	S
"		7	S
		8	S
	3 cc. intramuscularly	9	D_3‡
		1-0	D_3
		1-1	D_3
Untreated controls		1-2	D_3
		1-3	D_3
		1-4	D_3
		1-5	D_3
		1-6	S

All guinea pigs were given 0.05 cc. of a 2 per cent virus suspension in each nostril.

* Serum given 2 hours before virus.

† S = survived without showing signs of disease.

‡ D_3 = died on 3rd day after inoculation, with evidence of scratching some part of the head.

pseudorabies by way of the nose, and (*b*) in view of the fact that the intramuscular injection of an even larger amount of immune serum (about 1 cc. per 100 gm.) failed to prevent infection, the action of the nasally instilled serum was necessarily a local one. The possibility of greater absorption of a diffusible agent into the cerebrospinal fluid

the serum survived without showing any signs of disease, and demonstrate further that definite protection is evident at 5 hours, while an occasional guinea pig may perhaps still be resistant at 24 hours. All the animals which were treated either with 1 per cent tannic acid or the 0.1 per cent neutral acriflavine died with typical signs of pseudorabies, as did two of the three untreated ones.

There are several points in this experiment which may be stressed. One, the relatively short duration of the protective action of nasally instilled immune serum, may be accounted for by the probable washing out effect of the nasal secretions, which is in agreement with observations showing that *in vitro* minced, susceptible tissue, rendered refractory to infection by exposure to antiviral serum, may again become susceptible after repeated washing (5). It has also been recorded (11) that the testes from a rabbit actively immune to Virus III may, by washing, be rendered susceptible to infection *in vitro*. The negative results with neutral acriflavine are of interest since it may mean that its action in the nose differs from that in the skin, or that its protective effect may be limited only to certain viruses, both possibilities being worthy of further study.

Influence of Quantity of Serum Administered.—When 0.5 cc. of serum is instilled into each nostril of a guinea pig, possibly the greater portion of it is lost by sneezing or swallowing. Yet, when half that amount of immune serum, *i.e.*, 0.25 cc., was used in the preliminary experiments and the dose of virus was equally large, protection rarely followed. In view of the results of the preceding experiments, it appeared possible that this difficulty was due mainly to the larger volume or amount of virus which either spread more easily beyond protected zones or persisted after the effect of the serum had worn off. In the present test the effectiveness of the 0.25 and 0.5 cc. doses was compared in two groups of four guinea pigs (Table III). The dose of virus, which was 0.05 cc. of a 5 per cent suspension for each nostril, proved less infective than anticipated, since only two of the four controls developed the disease and died. It is noteworthy, however, that none of the serum-treated guinea pigs, which received this dose of virus 4 hours after the serum, showed any signs of disease.

Attempts at Prolonging the Duration of the Protective Effect of Nasally Instilled Immune Serum.—Since the relatively rapid loss of the refractory state induced by the nasal instillation of immune serum may

infection with vaccinia virus. The neutral acriflavine by itself was shown by them not to be virucidal, as evidenced by the fact that upon dilution of such an inactive mixture to a point at which the concentration of acriflavine became too dilute to be effective, they obtained a suspension which was again infective. The

TABLE II

Duration of Local Protective Action of Antipseudorabies Serum When Administered by the Nasal Route and Effect of Certain Chemicals

	Treatment	Interval between treatment and virus	Guinea pig No.	Result
		hrs.		
	Antipseudorabies swine serum 0.5 cc. in each nostril	2	1-7	S
			1-8	S
			1-9	S
			2-0	S
		5	2-1	S
			2-2	S
			2-3	S
			2-4	D_3
		24	2-5	S
			2-6	S
			2-7	D_3
			2-8	D_4
Controls	1% tannic acid 0.5 cc. in each nostril	2	2-9	D_3
			3-0	D_3
	0.1% neutral acriflavine 0.5 cc. in each nostril	3	3-1	D_3
			3-2	D_3
			3-3	D_3
			3-4	D_3
			3-5	D_3
	None	—	3-6	D_3
			3-7	D_3
			3-8	D_3
			3-9	S

All guinea pigs received 0.05 cc. of a 2 per cent virus suspension in each nostril.

eight chemically treated guinea pigs and three untreated ones received pseudorabies virus intranasally along with the serum-treated animals.

The results (Table II) confirm the observations of the previous experiment, in that all the guinea pigs receiving virus 2 hours after

the serum survived without showing any signs of disease, and demonstrate further that definite protection is evident at 5 hours, while an occasional guinea pig may perhaps still be resistant at 24 hours. All the animals which were treated either with 1 per cent tannic acid or the 0.1 per cent neutral acriflavine died with typical signs of pseudorabies, as did two of the three untreated ones.

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		hrs.		
	Antipseudorabies swine serum 0.5 cc. in each nostril	2	1-7	S
			1-8	S
			1-9	S
			2-0	S
		5	2-1	S
			2-2	S
			2-3	S
			2-4	D_3
		24	2-5	S
			2-6	S
			2-7	D_3
			2-8	D_4
Controls	1% tannic acid 0.5 cc. in each nostril	2	2-9	D_3
			3-0	D_3
			3-1	D_3
			3-2	D_3
	0.1% neutral acriflavine 0.5 cc. in each nostril	3	3-3	D_3
			3-4	D_3
			3-5	D_3
			3-6	D_3
	None	—	3-7	D_3
			3-8	D_3
			3-9	S

All guinea pigs received 0.05 cc. of a 2 per cent virus suspension in each nostril.

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The results (Table II) confirm the observations of the previous experiment, in that all the guinea pigs receiving virus 2 hours after

Tests for Active Immunity in Protected Guinea Pigs.—Twelve guinea pigs which, as a result of the nasal instillation of immune serum, proved to be refractory to a generally lethal test dose of pseudorabies virus, were retested with the same dose and by the same route 10 to 15 days later to determine whether or not they had acquired any

TABLE IV

Effect of Mixing Glycerol or Liquid Paraffin with Immune Serum on Its Protective Action in the Nose

Treatment (0.5 cc. in each nostril)	Amount of 1% virus suspension in each nostril	Guinea pig No.	Result
	cc.		
None	0.025	5-2	S
		5-3	S
		5-4	S
		5-5	D_4
	0.05	5-6	D_4
		5-7	D_3
Immune serum—3 parts + Saline—1 part	0.05	5-8	S
		5-9	S
		6-0	S
		6-1	S
Immune serum—3 parts + Liquid paraffin—1 part	0.05	6-2	D_3
		6-3	S
		6-4	S
		6-5	S
Immune serum—3 parts + Glycerol—1 part	0.05	6-6	S
		6-7	S
		6-8	S
		6-9	D_3

Virus given 2 hours after treatment.

active immunity. As all of them developed typical pseudorabies and died, it could be assumed that the primary treatment had protected them completely from the effects of the virus so that no active immunity ensued.

Experiments with Mice and Equine Encephalomyelitis Virus (Eastern Strain).—The purpose of the following experiments was to determine

be attributable to the fact that it is washed away by the nasal secretions, several attempts were made to determine whether certain agents may help to retain the serum in the nose for a longer time.

In the first experiment, three parts of immune serum were mixed with one part respectively of saline, glycerol, or liquid paraffin, each mixture (0.5 cc. for each nostril) was given to a group of four guinea pigs, and the virus was administered 2 hours after treatment. The results, shown in Table IV, indicate that while glycerol and liquid paraffin did not inhibit the action of the serum, they exerted no appreciable effect, since all the guinea pigs treated with the serum-

TABLE III

Relation between Quantity of Antipseudorabies Serum Administered Intranasally and Susceptibility to Infection

Treatment Swine antipseudorabies serum	Guinea pig No.	Result
0.25 cc. in each nostril	4-0	S
	4-1	S
	4-2	S
	4-3	S
0.5 " " " "	4-4	S
	4-5	S
	4-6	S
	4-7	S
None—controls	4-8	D_4
	4-9	D_3
	5-0	S
	5-1	S

0.05 cc. of 5 per cent virus suspension in each nostril. Interval between serum and virus—4 hours.

saline mixture survived, while one of the four in each of the other two groups developed the disease and died. Eight guinea pigs were treated with immune serum mixed with sufficient agar (about 0.66 per cent) to make a gelatinous mass and tested with virus 6 and 24 hours later, but they behaved as did the controls, all succumbing in average time. Dried serum mixed with petrolatum was totally ineffective. Eight guinea pigs were treated with immune serum in the usual manner and within 20 to 30 minutes each was given 0.25 cc. of 0.66 per cent tannic acid per nostril to determine the possible effect of precipitating the serum *in situ*. Virus was given 6 and 24 hours later. No advantage over the ordinary serum-treated guinea pigs was secured (Table V).

whether the phenomenon of local immune serum protection against infection by the nasal route occurred with another virus and in another host.

TABLE VI

Effect of Nasal Instillation of Immune Serum on the Susceptibility of Mice to Infection with Eastern Equine Encephalomyelitis Virus by the Nasal Route

Experiment	Treatment (0.05 cc. intranasally)	Interval between serum and virus	Amount of virus		No. of mice used	No. survived
			cc.	Dilution		
		hrs.				
I	Immune serum	2	0.02	1:200	6	6
		4	"	"	6	4
Virus suspension in broth	None	—	"	"		
			"	"	3	0
			"	1:1,000	3	0
			"	1:10,000	3	0
			"	1:100,000	3	2
			"	1:1,000,000	3	3
II	Immune serum	2.5	0.03	1:200	6	1
		4	"	"	6	1
Virus suspension in broth	Normal serum	2.5	"	"		
	None	—	"	"	6	0
			"	"		
			"	1:1,000	6	0
			"	1:10,000	3	0
III	Immune serum	2.5	0.02	1:200	3	1
		2.5	0.03	"	6	5
Virus suspension in saline	Normal serum	2.5	0.02	"	6	4
	None	—	"	"	6	1
			"	"		
			"	1:1,000	3	0
			"	1:10,000	3	2
					3	3

Eastern equine encephalomyelitis virus given to mice intranasally produces a fatal encephalomyelitis in 3 to 5 days. Twelve mice were each given 0.05 cc. of rabbit hyperimmune serum by placing drops of the serum on their nasal orifices and allowing them to aspirate. The test dose of virus was 0.02 cc. (similarly administered) of a 1:200 dilution of centrifuged fresh mouse brain suspension in broth. Since this virus can produce encephalitis by routes other than the nasal,

TABLE V

Attempted Prolongation of Protective Effect of Antipseudorabies Serum in Nose by Combination with Various Agents

Experiment	Treatment	Interval between treatment and virus	Guinea pig No.	Result
		hrs.		
I	Antipseudorabies swine serum alone	6	7-0	S
			7-1	S
			7-2	D_3
			7-3	D_3
	Immune serum in agar jelly	6	7-4	S
			7-5	S
			7-6	S
			7-7	D_5
	Dry immune serum in petrolatum	6	7-8	D_3
			7-9	D_4
			8-0	D_3
			8-1	D_3
	None	—	8-2	D_3
			8-3	D_4
			8-4	D_4
			8-5	S
II	Immune serum in agar jelly	6	8-6	S
			8-7	D_3
			8-8	D_3
			8-9	D_3
		24	9-0	S
			9-1	S
			9-2	D_3
			9-3	D_3
	Immune serum followed in 20 min. by 0.66% tannic acid	6	9-4	S
			9-5	S
			9-6	D_2
			9-7	D_4
		24	9-8	S
			9-9	D_3
			1-00	D_3
			1-01	D_5
	None	—	1-02	D_2
			1-03	D_3
			1-04	D_3
			1-05	D_3

0.05 cc. of a 2 per cent virus suspension in each nostril.

nasal route. If, as is generally assumed, the virus comes in direct contact with nervous elements in the nose, the phenomenon of specific local protection in this region is particularly noteworthy in the case of pseudorabies in the guinea pig, since the immune serum is practically devoid of any antiviral action when introduced with the virus directly into the brain, and it would appear, therefore, that in this instance, at least, the capacity or mode of protection may be different in peripheral and central nervous tissues. The subject is obviously one calling for further study.

The protective effect of immune serum in the nose begins immediately after instillation, is still evident 5 hours later, and usually is entirely dissipated by the end of 24 hours. Attempts to prolong its effect by incorporating with the serum substances such as glycerol, liquid paraffin, semisolid agar, petrolatum, and by precipitating the serum *in situ* with tannic acid proved unsuccessful.

SUMMARY

1. Immune serum instilled intranasally in guinea pigs has protected them from infection with lethal amounts of pseudorabies virus by the nasal route. The same effect was obtained in mice with immune serum against the virus of equine encephalomyelitis (Eastern strain).

2. The protective effect of the immune serum in the nose begins at the time of instillation, is still evident 5 hours later, and usually has disappeared by the end of 24 hours.

3. Attempts to prolong the local effectiveness of immune serum by means of several devices were unsuccessful.

4. The action of the immune serum appears to be a local one in the nose, in view of the fact that the administration of even larger amounts of it intramuscularly failed to protect guinea pigs against infection with pseudorabies by the nasal route.

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the test dose was only 0.02 cc. in order to prevent as far as possible its being aspirated into the lungs. One group of six mice received the virus 2 hours after treatment and the other group after an interval of 4 hours. The infectivity of the virus suspension by the nasal route was simultaneously titrated on mice of similar age. As shown in Table VI, while all the control mice which received 0.02 cc. of the 1:200, 1:1,000, and 1:10,000 dilutions of the virus, and one of three of the 1:100,000 group developed a fatal encephalitis, none of the six mice treated 2 hours before receiving virus showed any signs of disease and only two of the six mice treated 4 hours before infection developed encephalitis. The ten protected, surviving mice were tested for active immunity 18 days later and all succumbed, indicating, as in the case of pseudorabies, the lack of an active immunity.

A suspension of the virus in broth has been found to be considerably more encephalitogenic in mice than one in saline, and when 0.03 cc. instead of 0.02 cc. of virus suspension in broth was used as the nasal test dose, protection rarely occurred (Experiment II, Table VI, and others). With saline suspensions of virus, protection was shown with both doses. It should also be stated that while in the case of pseudorabies the intramuscular injection of 3 cc. of immune serum to guinea pigs weighing about 300 gm., or the equivalent of 10 cc. per kilo, failed to protect them against nasal infection with a dose in the minimal effective range, four 20 gm. mice, each receiving 1 cc. of immune serum intraperitoneally, or the equivalent of 50 cc. per kilo, 4 hours before infection, were protected against a lethal amount of equine encephalomyelitis virus given by the nasal route.

From these experiments it appears that while passive systemic immunity against nasal infection, that is, against invasion of virus by the nasal route, is not an impossibility, it is nevertheless difficult to achieve probably because of the relatively large amount of antibody required in larger hosts.

DISCUSSION

The purpose of this communication is to present evidence that the nasal mucosa and the nerve endings in it may be protected temporarily from infection with certain viruses by a preliminary flushing or spraying of these tissues with immune serum, and that when these primary receptor cells for the virus are so blocked or protected, the virus does not invade the rest of the organism and disease does not ensue. That the action of the immune serum thus introduced in the nose is a local one is apparent from an experiment in which larger amounts of the same serum given systemically failed, in the case of pseudorabies virus, to protect guinea pigs against infection by the

PROTECTIVE ACTION OF CERTAIN CHEMICALS AGAINST INFECTION OF MONKEYS WITH NASALLY INSTILLED POLIOMYELITIS VIRUS*

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The virus of poliomyelitis is believed by most investigators to enter the human body usually by way of the nose, and in view of the fact that the disease can be produced readily in *Macacus rhesus* monkeys by this route (1), the experiments of recent years have been concerned for the most part with production of resistance in them to nasal infection.

Active and passive immunization have thus far failed to provide a means for the regular protection of monkeys against infection with poliomyelitis virus by way of the nose (2–6). Another approach to the problem of preventing poliomyelitis was made possible by recent studies on the effect of the nasal instillation of certain chemical agents on the susceptibility to infection with viruses by the nasal route.

Olitsky and Cox (7), in 1934, showed that mice which received a number of nasal instillations of tannic acid or alum were rendered resistant to subsequent infection with equine encephalomyelitis virus by the nasal route. Shortly thereafter, Armstrong (8) reported a similar effect of sodium alum in the prevention of nasal infection of mice with the virus of St. Louis encephalitis, and while the present investigation was in progress, Armstrong and Harrison (9) reported experiments on the prevention of poliomyelitis in monkeys. They found that "the instillation of sodium aluminum sulphate, 4 per cent, into the nostrils of monkeys resulted in the survival of 17 from a group of 23 animals, while only 3 from a group of 19 nonprepared controls survived similar intranasal inoculation with poliomyelitis virus." Of the 17 surviving, alum-treated monkeys one

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9. Olitsky, P. K., and Cox, H. R., *Science*, 1934, **80**, 566.
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dose of virus, and frequent observations were made of the physical conditi
Approximately 80 per cent of normal monkeys given two doses of virus as d
scribed above developed paralysis, which in most instances was progressive, lea
ing to death, although the monkeys were as a rule killed by ether inhalation whe
they became prostrate. The monkeys which failed to develop paralysis showed
neither fever nor physical signs which could be attributed to poliomyelitis, and
when retested by the same method a month or two later almost always suc-
cumbed with typical poliomyelitis. It appears, therefore, that with the virus
and monkeys used the failure of some few animals (about 20 per cent) to develop
paralysis after one test is probably due more to the method or the potency of the
virus rather than to any inherent resistance on the part of the animals.

In view of the fact that an occasional monkey in an experiment failed to
contract the disease, it was necessary even in preliminary tests to use more than
one or two monkeys. With three monkeys as controls, two always developed
paralysis and sometimes all three; when four were used, three of them, as a rule,
and sometimes four showed poliomyelitis; frequently more than four controls
were employed for an experiment.

Treatment with Tannic Acid or Sodium Alum.—Solutions of tannic acid
(tannin) and sodium alum, C.P., ($Al_2Na_2(SO_4)_4 \cdot 24\ H_2O$) were made up in the
desired concentrations in distilled water and kept at room temperature. 1.5 cc.
of the solution to be tested was instilled into each nostril from a pipette or
syringe fitted with a rubber urethral tip and administered with the same tech-
nique as the virus. The frequency with which the chemicals were given and the
time relationship between treatment and instillation of virus varied in different
experiments.

EXPERIMENTAL

Protective Action of Nasal Instillations of Sodium Alum and Tannic Acid.—Previous experiments carried out in this laboratory indicated that tannic acid or alum when used in concentrations of 0.5 to 1 per cent was capable of protecting mice against infection with the virus of equine encephalomyelitis by the nasal route; it was furthermore pointed out (7) that treatment had to be continued for at least 3 days to obtain optimum results.

In Table I is shown a series of tests in which similar concentrations of tannic acid and alum were used in monkeys subsequently given poliomyelitis virus intranasally. In all but one of the groups the tannic acid or the alum was given once a day for 3 days and again on the days of infection, 6 hours before the instillation of virus. The group of monkeys treated with 0.8 per cent tannic acid received instillations three times a day for 2 days, two instillations 4 hours apart on the 3rd day and 4 hours later, the first dose of virus, after which no more of the chemical was administered.

developed partial paralysis of the hind legs and 7 "ran a course of fever, beginning from 5 to 21 days following the first inoculation and lasting from 6 to 13 days, which seemed probably due to poliomyelitis."

The purpose of the present investigation was to answer as far as possible the following: (*a*) Has nasal instillation of tannic acid or sodium alum, in proper concentration, any effect on the susceptibility of monkeys to infection with poliomyelitis by the nasal route and if it should be found to have a preventive action, (*b*) what is the time required for the refractory state to be induced? (*c*) What is the duration of the refractory state, and (*d*) is the resistance the result of an action of these chemicals on the virus or on the tissues? (*e*) Can the refractory state be maintained over a period of weeks by daily instillations, or does it disappear in spite of continued treatment, and (*f*) what is the effect of beginning treatment soon after, rather than before, the virus is administered? (*g*) Does specific antiviral immunity develop in the refractory animals, and (*h*) can these chemicals be safely instilled in the nose of man?

Methods and Materials

Virus.—The M.V. or mixed virus strain which has undergone many monkey passages in this Institute was used in all tests. The spinal cords and medullae of nasally instilled monkeys which were sacrificed at the earliest possible time after the development of complete paralysis, were preserved in 50 per cent glycerol-saline solution and used within a month or two. Pieces of tissue from three or four monkeys were pooled, and after removing the meninges washed in three changes of physiological saline solution, the cords were weighed and ground with sand, adding sufficient saline solution to make a 10 per cent suspension. This suspension was centrifuged for 2 to 3 minutes at low speed, sufficient to sediment the sand and large particles. After pouring off the supernatant liquid, the resultant milky tissue suspension was used for nasal instillation. 1 cc. of this fluid was introduced into each nostril by means of a tuberculin syringe to which was attached a well fitting rubber urethral tip. The monkey's head was tilted downward, the fluid expelled quickly from the syringe, then drawn back into it and expelled once more, closing the nostrils with the fingers upon withdrawing the syringe. A fresh virus suspension was prepared 48 hours later and the same dose repeated. No lavage preceded the administration of the virus.

Experimental Disease in Normal Animals.—*Macacus rhesus* monkeys of the usual size, *i.e.* about 2 to 3 kilos, were used in all the experiments but one, in which an effort was made to test particularly young animals weighing about 1 kilo. Rectal temperatures were taken daily for at least a month after the first

The results indicate that concentrations of tannic acid and alum, applied in a manner capable of protecting mice against nasal infection with equine encephalomyelitis virus, were ineffective in protecting monkeys against infection with poliomyelitis virus by the nasal route.

When, however, the concentration of alum and tannic acid was increased to 3 or 4 per cent, a distinctly beneficial protective effect was obtained. Table II shows the results of three different experiments. Of 27 monkeys treated with either 3 or 4 per cent alum or tannic acid, only four developed poliomyelitis; while of 28 control monkeys simultaneously infected with the same virus suspensions, 22 became paralyzed. In Experiments C and E none of the treated monkeys developed the disease, while in Experiment D, in which the virus suspension might have been unusually potent (all five control monkeys succumbed), four of the eight treated monkeys exhibited poliomyelitis. It may be worth while to note that in Experiment C monkeys weighing about 1 kilo and probably less than a year old were used. In Experiment E one group of four monkeys received daily nasal instillations of 4 per cent alum over a period of 13 days; this was done to determine whether the refractory state once developed, as may be assumed from the other experiments, might disappear within a few days in spite of continued treatment. However, all of the four monkeys so treated proved resistant, indicating that the protective effect does not disappear too rapidly, at least not while the treatment is being continued daily. Among the 23 treated monkeys which resisted infection, there was none with any evidence of a mild or abortive attack; the majority showed no fever, while occasional rises of temperature among others could not be attributed to poliomyelitis. In other words, the protective effect in the monkey was either complete or not at all present.

Conditions Influencing Development of Refractory State.—To ascertain the optimum conditions for inducing in monkeys the refractory state to nasal infection with the virus, as well as to recognize its limitations, it was desirable to know something of the manner in which this state was brought about. Clearly, the first question to arise concerns the effect of these chemicals on the virus itself, in order to determine the rôle of tannic acid or alum remaining adherent to the

TABLE I

Influence of Tannic Acid and Sodium Alum in Concentrations Effective in Mice on Susceptibility of Rhesus Monkeys to Infection with Poliomyelitis by Nasal Route

Experiment	Substance used for nasal instillation	Concentration	Mode of treatment and infection—*days*						Monkey No.	Result
			1	2	3	4	5	6		
		per cent								
A	Tannic	0.4				V*		V	1	Dead 26th day. Poliomyelitis?
	acid		T	T	T	↑		↑		
	(T)					T		T	2	Fever 4, par. 7, prostr. 8†
									3	Remained well
		0.8				V		V	4	" "
				T	T	↑			5	Fever 6, par. 10, prostr. 11
				t.i.d.	t.i.d.	T				
						b.i.d.			6	" 6, " 9, " 11
	Untreated					V		V	7	" 8, " 12, " 12
	controls								8	" 7, " 11, " 13
									9	Remained well
B	Sodium	0.5				V		V	1-0	Fever 5, par. 9, prostr. 10
	alum					↑		↑	1-1	" 5, " 10, " 12
	(A)		A	A	A	A		A		
									1-2	Remained well
		2.0				V		V	1-3	Fever 5, par. 10, prostr. 11
						↑		↑	1-4	" 6, " 10, " 11
			A	A	A	A		A		
									1-5	" 4, " 8, " 10
	Untreated					V		V	1-6	" 4, " 8, " 8
	controls								1-7	" 7, " 12, " 13
									1-8	" 5, " 8, " 9
									1-9	Remained well

Par. 7 = first signs of paralysis 7 days after first dose of virus.

Prostr. 8 = complete paralysis of all extremities with subnormal temperature, *i.e.* prostrate, 8 days after first dose of virus.

* V = virus intranasally.

† Fever 4 = distinct rise of temperature (about 105°F. or more) above previous level, 4 days after first dose of virus.

A 20 per cent suspension of poliomyelitis virus in 0.85 per cent NaCl was divided into two parts; one was mixed with an equal part of 0.85 per cent NaCl, and the other with an equal amount of 4 per cent sodium alum. Immediate gross flocculation occurred in the alum mixture. Both were left at room temperature for 30 minutes, and then given to monkeys intranasally in the usual manner. Of two monkeys treated with the alum mixture, one succumbed to poliomyelitis, and of the four receiving the saline suspension two developed the disease.

There is no apparent direct action of these chemicals on the virus which would account for the refractory state. That the protective action is brought about by a local, rather than a systemic, effect of these substances has been shown in experiments with mice, in which the nasal instillation of tannic acid prevented infection when equine encephalomyelitis virus was given intranasally but not when it was injected intracerebrally (7). It was also indicated in the same communication (7) that mice had to be treated several times a day for at least 3 days to obtain the optimum protective effect. In the present investigation it has already been shown that three single daily instillations of adequate concentrations of alum or tannic acid along with single instillations 6 hours before the virus proved effective. To determine whether 4 per cent alum might have any effect within 6 hours or even 48 hours, and for how long 3 days' treatment might be effective in preventing infection, the following experiments, summarized in Table III, were carried out.

Three monkeys were given one instillation of 4 per cent alum and the first dose of virus 6 hours later; the next day they received one alum treatment, and on the following day alum in the morning, followed by the second dose of virus 6 hours later. Since two of the three monkeys so treated developed poliomyelitis (three of four untreated controls succumbed), it was evident that the first dose of virus could not have been inhibited by the alum given 6 hours previously, and that the three alum treatments within 48 hours probably also had little effect on the second dose of virus. It was apparent, therefore, that whatever the changes produced by the alum leading to the refractory state, more than 2 days are required to bring them about.

In the next experiment (G) four monkeys were given nasal instillations of 4 per cent alum once a day for 3 days; the first dose of virus was administered 48 hours after the last alum instillation, and the second dose 96 hours later. As is indicated in Table III, the 3 days' treatment was insufficient to protect the animals against virus given 48 and 96 hours later. On the other hand, as will be

nasal mucosa in preventing infection. It has already been pointed out (4) that while tannic acid precipitates suspensions of poliomyelitis virus, the precipitates remain highly infective when injected intra-

TABLE II

Protective Action of Effective Concentrations of Sodium Alum and Tannic Acid

Experiment	Substance used for nasal instillation	Concentration	Mode of treatment and infection—*days*												No. of monkeys used	No developed poliomyelitis
			1	2	3	4	5	6	7	8	9	10	11	12	13	
		per cent														
C	Sodium alum (A)	4.0								A	A	A	V ↑ A		V ↑ A	4	0
	Untreated controls												V		V	9	8
D	Sodium alum (A)	3.0								A	A	A	V ↑ A		V ↑ A	4	2
		4.0								A	A	A	V ↑ A		V ↑ A	4	2
	Untreated controls												V		V	5	5
E	Sodium alum (A)	3.0								A	A	A	V ↑ A		V ↑ A	4	0
		4.0								A	A	A	V ↑ A		V ↑ A	4	0
		4.0								A	A	A	V ↑ A		V ↑ A	4	0
			A	A	A	A	A	A	A	A	A	A	A	A	A		
	Tannic acid (T)	4.0								T	T	T	V ↑ T		V ↑ T	3	0
	Untreated controls												V		V	14	9

cerebrally. To determine, however, whether or not such precipitation might interfere with the infectivity of the virus after nasal instillation, the following experiment was performed.

opment of the disease. Both questions have plainly a practical viewpoint, the first being designed to indicate whether or not there might be any danger of increasing the incidence of morbidity if alum were given to an individual already carrying the virus, and the second to show whether or not any benefit may be expected from treatment instituted soon after the virus gains entrance to the host.

Four monkeys were given the usual dose of virus intranasally and 3 hours later the first treatment with 4 per cent alum with another instillation of alum 24

TABLE IV

Effect of Alum Treatment Begun Soon after Nasal Instillation of Virus

Mode of treatment and infection—days						Monkey No.	Result	Remarks
1	2	3	4	5	6			
A	A	A	A	A	A	3-2	Remained well	Resistant to infection by nasal route 1 mo. later
↑		↑				3-3	" "	
V		V				3-4	Fever 6, par. 10, prostr. 12	
						3-5	" 6, " 12, " 13	
S	S	S	S	S	S	3-6	" 5, " 7, " 7	
↑		↑				3-7	" 7, " 11, " 14	
V		V				3-8	" 4, " 8, " 9	
						3-9	Remained well	Paralysis after reinfection by nasal route 1 mo. later
V		V				4-0	Fever 4, par. 9, prostr. 10	

A = 4 per cent sodium alum intranasally.
S = physiological saline intranasally.
V = poliomyelitis virus intranasally.
Other abbreviations as in Table I.

hours later. The next day the second dose of virus was given and again followed in 3 hours by alum; for the subsequent 3 days each monkey received one nasal instillation of alum a day. Four other monkeys were instilled with the same virus suspensions and, instead of alum, with physiological salt solution in the same amount and manner. These controls were included to determine whether nasal lavage in itself performed within 3 hours after virus administration may wash away enough of the infective agent to prevent onset of the disease. One untreated monkey received only the virus.

The results (Table IV) indicate first, that nasal lavage by itself, as carried out in this experiment, does not prevent the development

shown later (Table V), monkeys receiving five or more treatments retain their resistance for a month or two.

In view of the observations which indicate that the resistance induced by alum or tannic acid requires several days to develop and is not apparently the result of any direct effect on the virus, it seems more than probable that the action of these chemicals is on the tissues of the host.

TABLE III

Conditions Influencing Development of Refractory State

Experiment	Mode of treatment and infection—*days*							Monkey No.	Result
	1	2	3	4	5	6	7		
F					V		V	2-0*	Remained well
					↑		↑	2-1	Fever 4, par. 9, prostr. 11
					A	A	A	2-2	" 4, " 7, " 9
					V		V	1-6, 1-7, 1-8, 1-9	1-6, 1-7, 1-8 developed poliomyelitis (see Table I)
G	A	A	A		V		V	2-3	Fever 8, par. 11, prostr. 12
								2-4	" 6, " 8, " 10
								2-5	" 11, " 14, " 20
								2-6*	Remained well
					V		V	2-7	Fever 4, par. 7, prostr. 8
								2-8	" 8, " 11, " 12
								2-9	" 6, " 10, " 11
								3-0	" 5, " 10, " 11
								3-1	" 7, " 11, " 12

A = 4 per cent sodium alum intranasally.

See Table I for explanation of other abbreviations.

* Monkeys 2-0 and 2-6 succumbed to poliomyelitis when they were retested, again intranasally, 1 month later.

Effect of Treatment Begun Soon after Nasal Instillation of Virus.—The experiment shown in Table IV was performed in order to note (*a*) whether nasal instillations of alum have an early effect in favoring the invasiveness of the virus, and (*b*) whether the progression of the virus from the nose to the brain is sufficiently slow for treatment started soon after infection to have an inhibitory effect on the devel-

TABLE V

Persistence of Refractory State in Monkeys Treated with Effective Concentrations of Alum or Tannic Acid for 5 Days or Longer

Previous treatment	Monkey No.	Susceptibility to infection by nasal route. Time after last treatment: 1 mo.	2 mos.	3 mos.	Remarks
Single daily nasal instillation of 4% alum for 5 days	4-1	n.t.	n.t.	Poliomyelitis	4 normal controls—3 polio. Sera obtained just before test failed to neutralize
" "	4-2			"	
	4-3			"	
	4-4			"	
	4-5	Remained well	n.t.	n.t.	
	4-6	Poliomyelitis			
Same with 3% alum	4-7	"			
" "	4-8	"			
	4-9	Remained well	Poliomyelitis		
	5-0	" "	Remained well	Poliomyelitis	1st mo.—4 of 5 controls polio.
	5-1	" "	" "	"	
	5-2	Poliomyelitis			
Same with 4% alum	5-3	*Intracerebral			
	5-4	n.t.			
	5-5	"	Poliomyelitis		2nd mo.—all 4 controls polio.
	5-6	"	"		
4% alum for 13 consecutive days	5-7	Remained well	"		
	5-8	n.t.			
	5-9	"	Remained well	Poliomyelitis	3rd mo.—all 4 controls polio.
			" "	"	
Tannic acid for 5 days	6-0	Remained well			
	6-1	" "	Remained well	Remained well†	
	6-2	" "	Poliomyelitis		
	6-3	" "	"		
4% alum for 6 consecutive days	3-2	" "	Remained well	Poliomyelitis	
	3-3	" "	" "	Poliomyelitis	1st mo.—all 4 controls polio. 2nd mo.—same
			Poliomyelitis		
Total No. of monkeys tested.....		15	15	11	
Total No. of monkeys resistant...		11	7	1	

n.t. = not tested.

* This animal received as a test for resistance about 1 to 5 M.I.D. intracerebrally and succumbed to the experimental disease.

† After the third test, this monkey was bled and its serum showed antiviral bodies. It also resisted another intranasal test dose given at this time.

of poliomyelitis, since three of four monkeys so treated showed the disease; secondly, it may be assumed that alum treatment started soon after virus instillation does not increase the susceptibility of the monkey to infection, since only two of the four alum-treated monkeys exhibited poliomyelitis as compared with four of the five controls. One cannot readily attribute the resistance of the two treated monkeys to the alum which they received.[1] It is evident, moreover, from the results of the later tests on the susceptibility of the monkeys to infection, that the six alum treatments which they received appear to have rendered them resistant to the virus 1 month later, while their mate, saline-treated monkey, which similarly survived the first test, succumbed on reinoculation 1 month later.

Persistence of Acquired Resistance.—A subject for inquiry now was whether the monkeys which received two nasal instillations of virus (a total of 4 cc. of 10 per cent suspension) and resisted experimental infection as a result of treatment with either alum or tannic acid, acquired a specific active immunity as evidenced by the development of neutralizing or protective antibodies in their blood, and by the continued resistance to infection by either the intracerebral or nasal routes. It has recently been pointed out by Flexner (10) that normal monkeys, which for one reason or another fail to develop clinical poliomyelitis after nasal instillation of virus, do not show antibodies in their blood and are not immune to reinfection.

Four alum-treated monkeys which completely resisted two doses of poliomyelitis virus were bled 3 months later and submitted to infection by the nasal route. Their blood contained no neutralizing or protective antibodies (*i.e.*, 0.8 cc. of serum mixed with 0.2 cc. of a Berkefeld N filtrate of a 5 per cent suspension, or approximately 20 M.I.D., failed, after incubation, to prevent poliomyelitis) and all four developed the typical disease on reinoculation (Table V).

Evidently no active immunity was induced, and 3 months after treatment the monkeys were again fully susceptible to infection. It was still important to determine, if possible, how long the acquired

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tion were tried (dropper, nasal douche, spray) using approximately 1 to 3 cc. for each nostril. With the dropper and nasal douche, a certain amount of the solution invariably found its way into the pharynx giving rise to somewhat unpleasant taste sensations, which, however, did not occur when a spray was used. The latter also probably reached more remote parts of the nasal mucosa. Administration of the solutions by spray was therefore considered preferable.

For a few minutes after instillation of either 4 per cent tannic acid or sodium alum there is an open, astringent feeling, which is soon superseded by a sense of turgescence and slight irritation (tickling) accompanied by sneezing and the appearance of a clear mucoid discharge. This usually lasts for 30 minutes to 2 hours. The same symptoms reappear when the treatment is repeated on subsequent days, although sometimes with diminished intensity. There was no interference with the sense of smell. In some, the instillations were continued for 7 or 10 days without any harmful results. There appeared to be no appreciable difference between tannic acid and alum, and withal it was felt that the temporary slight unpleasantness was not sufficient to contraindicate its use.

SUMMARY AND DISCUSSION

In the present investigation evidence was obtained indicating that nasal instillations of suitable concentrations of sodium alum or tannic acid induce in *Macacus rhesus* monkeys resistance to the development of poliomyelitis when the virus is introduced by the nasal route. It was found that apparently different concentrations of these chemicals are required to exert this type of protective effect in different hosts or against different viruses, for while mice are readily protected against nasal infection with equine encephalomyelitis virus by 0.5 per cent solutions of tannic acid or alum (7), monkeys require at least 3 per cent solutions to become resistant against poliomyelitis. Experiments designed to elucidate the development of this refractory state in monkeys indicate that it is a result of the action of these chemicals not upon the virus but rather upon the tissues of the host (probably the olfactory mucosa); further evidence in favor of this hypothesis may be found in the observation that several days of treatment are required before resistance is induced. While one nasal instillation a day for 3 days proved effective when additional treatments were given on the days of virus administration, there was no protection against infection when the virus was given 48 and 96 hours after the last treatment. The resistance which was demonstrable when the virus was instilled intranasally on the 4th and 6th

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In the present investigation evidence was obtained indicating that nasal instillations of suitable concentrations of sodium alum or tannic acid induce in *Macacus rhesus* monkeys resistance to the development of poliomyelitis when the virus is introduced by the nasal route. It was found that apparently different concentrations of these chemicals are required to exert this type of protective effect in different hosts or against different viruses, for while mice are readily protected against nasal infection with equine encephalomyelitis virus by 0.5 per cent solutions of tannic acid or alum (7), monkeys require at least 3 per cent solutions to become resistant against poliomyelitis. Experiments designed to elucidate the development of this refractory state in monkeys indicate that it is a result of the action of these chemicals not upon the virus but rather upon the tissues of the host (probably the olfactory mucosa); further evidence in favor of this hypothesis may be found in the observation that several days of treatment are required before resistance is induced. While one nasal instillation a day for 3 days proved effective when additional treatments were given on the days of virus administration, there was no protection against infection when the virus was given 48 and 96 hours after the last treatment. The resistance which was demonstrable when the virus was instilled intranasally on the 4th and 6th

refractory state persisted in the adequately treated monkeys, which did not develop poliomyelitis on the first test.

Accordingly, most of the refractory monkeys were retested at monthly intervals until practically all developed poliomyelitis (Table V). Needless to say, a suitable number of normal monkeys was included in each test. It should be stated here that normal monkeys, which remain well after the nasal test with virus, almost always (only one exception was observed during the present investigation) succumb when retested 1 month later (see Flexner 10)) and not infrequently even more rapidly and regularly than monkeys which are receiving the virus for the first time.

It seems highly significant that of 15 refractory monkeys retested after 1 month, 11 failed to develop poliomyelitis; 10 of these 11 were tested again at 2 months and five came down with the disease while five were still resistant. Including monkeys of other series, 15 refractory animals were subjected to intranasal instillation with virus after a period of 2 months, of which seven were still found to be resistant. Of 11 monkeys similarly tested after 3 months, 10 developed poliomyelitis.

In a previous experiment (Table III) it was shown that three treatments with 4 per cent alum are either insufficient to induce resistance, or when such protection is conferred it does not persist for 48 to 96 hours. In the present tests (Table V), it appears that five or more treatments with 3 or 4 per cent sodium alum or 4 per cent tannic acid, given on as many days, protect the majority of monkeys (about 73 per cent) for at least a month, and almost half of them (about 47 per cent) for at least 2 months.

Effect of Nasal Instillations of Tannic Acid and Alum in Man.— The results of the preceding experiments suggested that similar procedures might perhaps be tried in man if the chemicals used, *i.e.* tannic acid and alum, prove neither too irritating nor possibly harmful on prolonged instillation. Tannic acid and alum have been used in human therapeutics for many years without any obviously harmful results; both have been used even in the solid state for controlling epistaxis.

To determine the symptoms resulting from the continued nasal instillation of 4 per cent tannic acid or sodium alum, five volunteers took either one or the other preparation daily over a period of 5 days or more. Various methods of instilla-

viduals who are subject to frequent attacks of common cold may perhaps be profitable.

CONCLUSIONS

1. Sodium alum or tannic acid instilled intranasally in proper concentration and over a period of several days protects monkeys against subsequent infection by the nasal route with the virus of poliomyelitis.

2. While mice are protected against equine encephalomyelitis virus when 0.5 per cent solutions of either tannic acid or alum are used, the minimal concentration effective in preventing poliomyelitis in monkeys is 3 per cent, indicating different requirements as regards variations either in the host or the virus.

3. Treatment over a period of at least 3 days is required to induce resistance which, once developed, does not disappear quickly when the nasal instillations of the chemical are repeated daily.

4. Treatment for 3 days does not in itself confer sufficient resistance to endure for 48 to 96 hours, but when it is continued for 5 days or more the majority of monkeys (11 of 15) are still refractory about a month later, and about half the number (seven of 15) 2 months later, while at the end of 3 months normal susceptibility again prevails.

5. Neither tannic acid nor alum acts directly on the virus in a manner to prevent it from causing poliomyelitis by way of the nose; the action of these chemicals is rather on the tissues of the host (olfactory mucosa?), the changes leading to the refractory state requiring several days to be brought about.

6. Sodium alum does not have an early or primary effect favoring invasiveness of the virus, for when treatment is begun soon after the administration of the virus, the incidence of the disease is either unchanged or perhaps even lower than among monkeys not so treated.

7. Monkeys, rendered refractory by treatment, fail to exhibit evidence of specific humoral or tissue immunity after exposure to the virus.

8. 4 per cent tannic acid or sodium alum can be given to human beings with only slight discomfort and no apparent harmful effects.

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days after the beginning of treatment was also found to be present a week later when the treatments were given daily, indicating that the refractory state can be maintained in this manner. It was observed, however, that monkeys which were given nasal instillations of alum or tannic acid for 5 days or more may retain the resistance they acquired for 1 or 2 months without any additional treatment (Table V); thus, of 15 refractory monkeys retested after 1 month, 11 were still resistant; seven of 15 monkeys were refractory when retested after 2 months, while only one of 11 failed to develop poliomyelitis after an interval of 3 months. These last results suggest that to maintain such a refractory state over a period of months it may be necessary to employ daily instillations only for about a week, with subsequent repetitions at intervals of several days or a week. An experiment performed to determine the effect of beginning alum treatment soon after, rather than before, the virus is administered, indicated that there is no danger from a possible early or primary effect enhancing the invasiveness of the virus. One cannot state with any certainty, however, whether or not in some of the monkeys so treated the development of poliomyelitis is inhibited or prevented. Finally, it should be stressed that treatment with alum or tannic acid either completely prevented the disease in monkeys, or else was entirely without effect. It is therefore not surprising that no evidence was obtained of development of specific, active immunity in monkeys rendered refractory by chemical treatment.

As far as could be ascertained, the nasal instillation of tannic acid or alum in man proved thoroughly innocuous beyond some local irritation for a short time. The desirability of testing this procedure as an aid in the prevention of poliomyelitis in man during the months of greatest incidence or during epidemics is quite apparent.

In conclusion, there may be broader implications in this approach to the prevention of certain infectious diseases transmitted naturally or experimentally by way of the nose. It seems desirable to make studies with other viruses transmitted by the nasal route, including those of influenza and the common cold. The evidence obtained in the present study that the chemically induced refractory state may persist in a large number of monkeys for a month or two after treatment over a period of 5 days or more, suggests that studies on indi-

SOME EFFECTS OF OVARIECTOMY DURING THE PERIOD OF DECLINING REPRODUCTIVE POWERS IN MICE

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(Received for publication, February 13, 1936)

While cancer of the breast is known to occur in all age periods following the prepubertal development of the mammary gland tissue, the most striking thing about its appearance in mice is that the lower range of standard deviation on the incidence curve is almost exactly coincident with the average age at which the breeding females begin to show signs of those involutory changes which mark the beginning of the breaking down of sexual function.

In mice of the dilute brown (Little dba) stock this process first indicates itself by the fact that following a period of uniformity and predictability in breeding behavior, during the 120 to 220 day period, the mice show ovarian changes which are reflected in the size of the litters born and in the inability of the mothers to nurse such young as are born alive. Both of these conditions grow progressively worse as the mice grow older. Since it is recognized that the ova are generated in the ovary, and since the mammary glands have been demonstrated to be largely dependent upon the ovary and its secretions for their normal functioning, it is not unreasonable to conclude that the reason, or at least one of the reasons, for the decline in fertility and the ability to lactate is attributable to some change or changes which are occurring in the ovaries.

That these changes are slow and that the part of them which affects the mammary gland tissue is in advance of the part which affects fecundity is indicated by the fact that the mice remain fertile until old age but that mammary function is lost before that time.

It is, therefore, reasonable to infer that the absence of or change in the ovarian secretions which in normal health stimulate the mammae

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Methods of Tabulation

In this paper three criteria of change in the cancer and death rates are used: (*a*) Percentage of cancer in the various age groupings. (*b*) Average life remaining to an individual alive at the beginning of each age class. (*c*) Deaths from cancer per hundred alive at the beginning of each age class.

The first of these differs from the third in that it records the percentage of those alive at the beginning of any age period which died of cancer in that, plus all following, age groups. The third records those dying of cancer during a single period among one hundred alive at the beginning of the period.

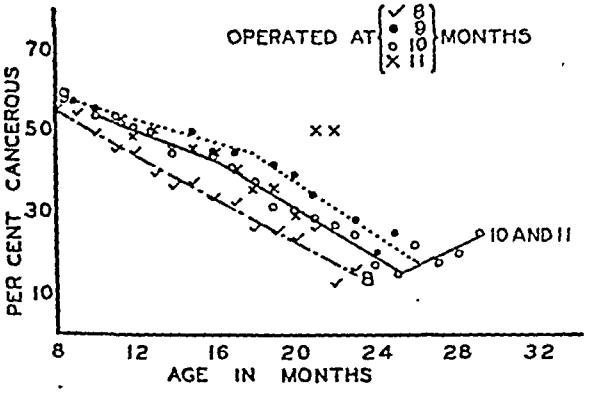

FIG. 1

The age groupings were divided into 30 day periods, which are designated in some places as months, in order to simplify the text.

In a short paper published in 1932[2] some of the effects of ovariectomy upon 195 breeding mice, operated at 7 months of age, were reported and discussed. It was found that this procedure greatly increased the average expectation of life and decreased appreciably the incidence of mammary tumors.

7 months was selected in that experiment because it marks the lower range of the standard deviation on the tumor incidence curve of this stock. This age seemed to be the most advantageous at which to

[2] Murray, W. S., *Science*, 1932, 75, 646.

is important in bringing about the change to malignancy in the mammary tissue.

With this in mind, the following experiment was attempted in an effort to demonstrate the effects upon tumor incidence of complete absence of the ovaries after a period of normal secretion.

Material and Methods

The mice used in this experiment were from the inbred dilute brown strain (Little dba). The records of the breeding females were taken from the tables published in 1934.[1] The ovariectomized females (364 in number) were mice which had been used in the breeding colony until they began to show signs of losing their reproductivity, as indicated by their increasing inability to give birth to normal sized litters, their inability to give birth to a normal proportion of living young and their failure to nurse a normal percentage of those young which were born alive.

These mice were separated from the males during the 8th, 9th, 10th, 11th and 12th months of life and were ovariectomized. After the operation they were segregated in pens containing five mice each and allowed to grow old. During this period they were examined once a week for tumors. As fast as they developed tumors or died from other causes they were autopsied and records taken.

The control group consisted of 551 females from the breeding stock. These were segregated in the same manner as the operated females and were kept under the same conditions of housing, examinations and care. In the tables and charts these mice are designated as old females.

The method of operating was as follows: The mice were anesthetized with pentabarbital sodium, 0.15 cc. of the solution obtained by dissolving 1½ grains of the salt in 15 cc. of distilled water, administered intraperitoneally.

The ovaries were then extirpated through bilateral incisions in the lumbar region, care being taken to remove all hair before cutting. Using this method, the glands are readily found caudal to the lower pole of the kidneys in young or thin mice. In those animals with some abdominal fat, the ovary is usually incorporated in a fat pad which seems to be characteristic of the locality in which the ovary is situated. In such cases, care must be taken in making the excision due to the large amount of blood which may be lost if the fat pad is cut deeply. No attempt was made to peel the ovaries from their capsules. The gland itself, the covering and the distal end of the oviduct were all removed. The incisions were closed by taking single stitches in the body wall and in the skin. With reasonable care there is little trouble from infection. The mice recover very quickly, becoming active as soon as they emerge from the anesthesia. Internal bleeding, in those animals in which the fat pad was damaged, is the largest cause of mortality.

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[1] Murray, W. S., *Am. J. Cancer*, 1934, 20, 573.

females, control females and operated females is illuminating since it shows the tendency in the breeding mice to rise steadily to 100 per cent in the oldest age group. This is the direct opposite of the behavior among the control and operated females which descend steadily until in the oldest age classes the mice are all free from tumors.

It will also be noticed that whereas the extreme age for breeding females was 21.5 months; in the control females it was 28 months and in the ovariectomized females 35 months. This shows an appreciable lengthening of life in both the experimental and the control classes.

Fig. 3 shows this lengthening of life even more graphically. Whereas the average length of life remaining to each breeding female alive

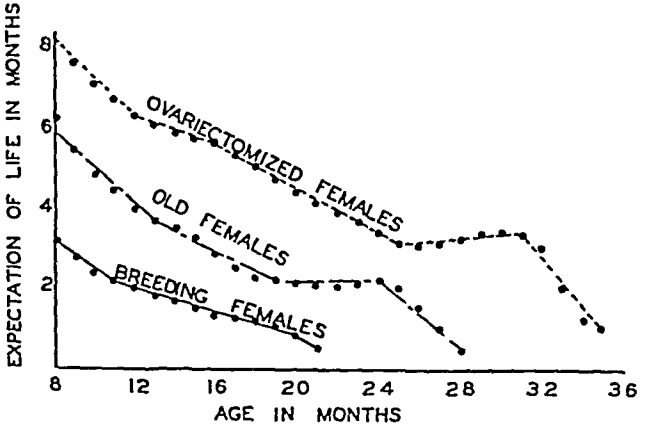

FIG. 3

at the beginning of the 8th month was 3.2 months, it was twice that for the old females and almost three times as much for the operated animals. As will be seen from the chart, these curves descend quite uniformly until at 21 months the average length of life remaining to the breeding females alive at the beginning of the period is 0.5 months; while it is 2+ months for the control females and 4+ months for the operated animals.

Fig. 4 shows the number of animals which developed tumor of the breast per hundred animals alive at the beginning of the various age periods. It will be noticed that while the tumor rate rises constantly and sharply in the breeding females, the curve for old females does not

influence the incidence of mammary tumors, since two-thirds or more of those which were to become cancerous would do so in the following 4 or 5 months.

It later became apparent[3] that at 7 months of age the breeding females of this stock are entering upon a period of decline in their ability to give birth and nurse their young. Additional mice were, therefore, operated at ages varying from 8 to 12 months as they individually showed signs of loss of sexual function. In all, 364 females were operated.

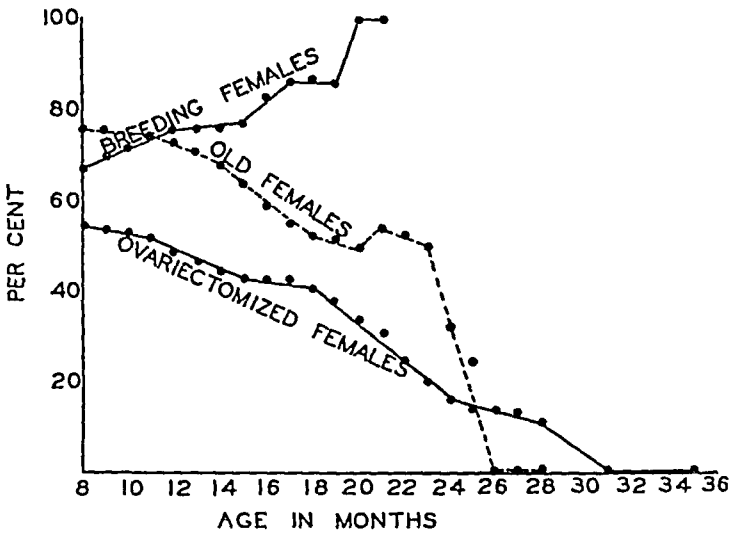

FIG. 2

That the incidence of cancer among the mice of this later experiment was approximately the same no matter at what age they were operated is shown by Fig. 1.

Inasmuch as the numbers were small when the animals were tabulated according to the month at which they were operated, especially in the older age classes to which relatively few lived, it was thought advisable to combine them, in order to show the general tendency of the effects of ovariectomy during the period when cancer of the mammary gland is most frequent in the breeding stock.

Fig. 2 which portrays the expectation of cancer in the breeding

[3] Murray, W. S., *Am. J. Cancer*, 1934, **20**, 584 (Table 14).

indicates the secretions of gestation and its sequelae in the breeding females are instrumental in the causation of cancer of the breast.

Owing to the variability of individual females in physiological age, as shown by the divergence in the times at which they undergo the

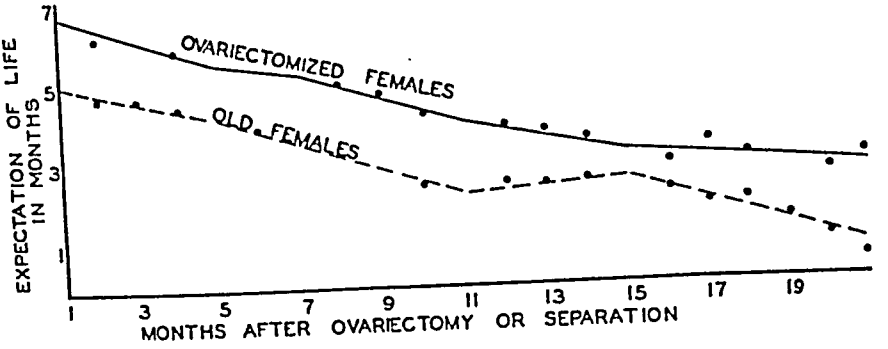

FIG. 6

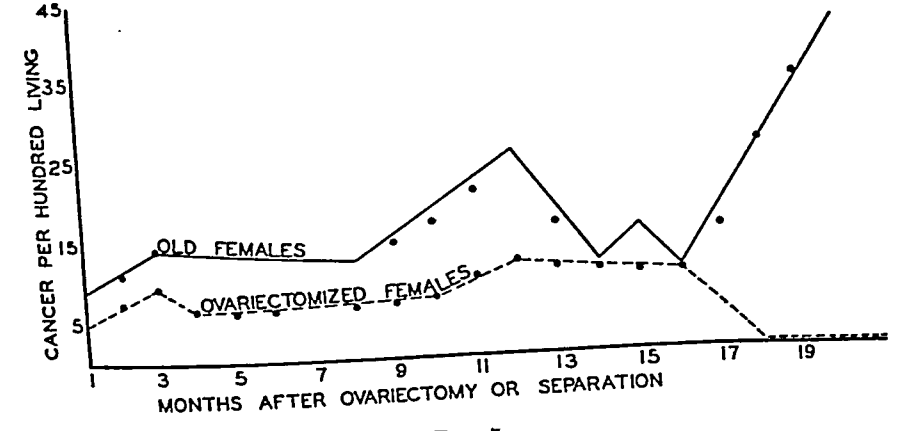

FIG. 7

decline in reproductive power, it was thought advisable to tabulate the same data using the time of separation or of operation as a starting point, thus establishing the approach of the decline in reproductive power as an index of physiological age. When this is done the expectation of tumors of the breast, in per cent, of those living at or beyond the beginning of the age period takes the form of Fig. 5.

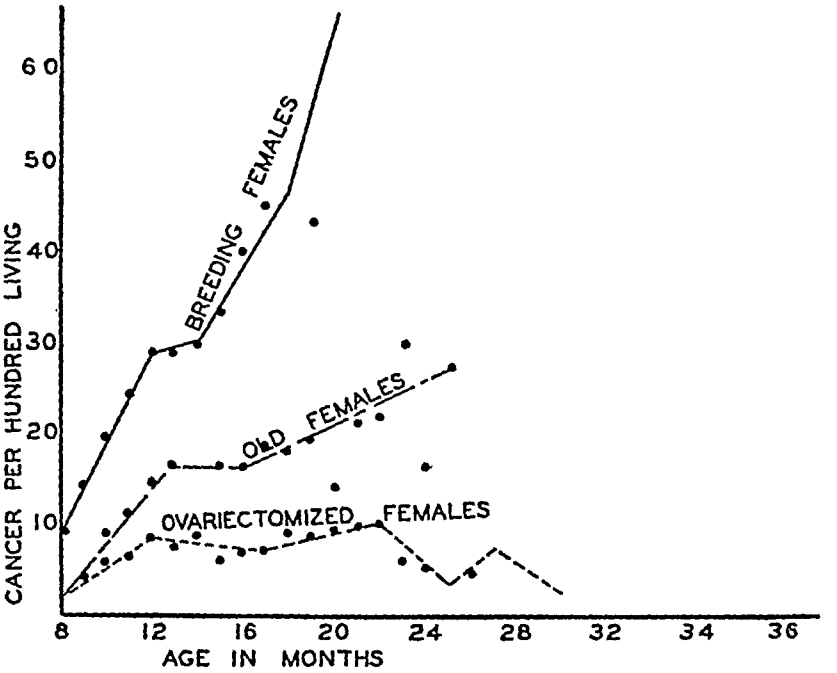

FIG. 4

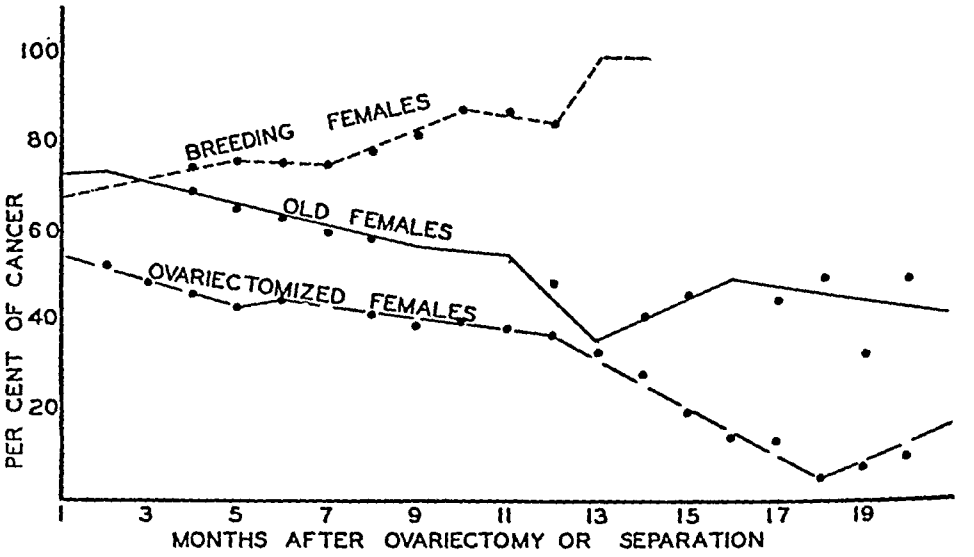

FIG. 5

rise so sharply and that the tumor rate per hundred living among the operated females is relatively constant in the various age classes. This

THE INFLUENCE OF INTRAUTERINE FACTORS ON THE FETAL WEIGHT OF RABBITS

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(Received for publication, March 20, 1936)

A lethal dwarf mutation in the rabbit, which appeared during the course of experiments designed for other purposes, has been the subject of a previous report (1) from this laboratory. These dwarfs, though born alive, all die within a few days. They are delicately formed and appear to be fully developed, but the bones of the calvarium are usually incompletely calcified. The observations on these animals appeared to indicate an incomplete recessive mutation which is characterized morphologically by a dwarfing effect and functionally by disorders in both heterozygous and homozygous individuals. The occurrence of this dwarf mutation emphasized the desirability of a general consideration of the factors influencing the birth weight of the rabbit. Studies pursued with this end in view had, however, the particular purpose of ascertaining to what extent environmental influences could account for the abnormally low weights of the animals denominated dwarfs. In approaching this problem, it was believed desirable first to determine whether intrauterine factors were operative in producing variations in fetal weight at or near term. An analysis of data accumulated with this end in view, together with certain collateral findings, forms the basis of the present report. Future communications will deal with the influence of gestation period, litter size, age and weight of the doe, season, and breed, on the birth weight of the rabbit.

Material and Methods

The observations on 475 fetuses carried by 71 pregnant rabbits form the basis of the present report. The majority of the does, all of which were bred in the colony, were the progeny resulting from miscellaneous hybrid matings, but a few

Here as in Fig. 2, the relationship of the three classes is the same. The number of tumors in breeding females rises, whereas it gradually falls in the controls and the females operated upon.

Turning to Fig. 6, which shows the average length of life remaining to individuals living 1 to 20 months after separation, the superiority of the castrates over the control females is not so marked as when tabulated in Fig. 3. The ovariectomized females do show, however, a uniform excess of 2 months over the controls.

In Fig. 7, as in Fig. 5, the cancer rate per hundred living at the beginning of each period remains uniform in the castrated females, while it rises slowly during the first 17 to 19 months after separation in the control females.

DISCUSSION

The inference that absence of, or changes in the ovarian secretions which in normal health stimulate the mammae, are important in bringing about the change to malignancy in the mammary tissue, is shown by the above charts to have some foundation in fact. The absence of the secretions accompanying gestation is shown to be instrumental in prolonging life and in decreasing the mammary tumor incidence; while if all ovarian activity is removed as in the castrated group, the effects in the same direction are even more striking. The tables demonstrate that these effects are apparent whether the criterion of age used be chronological or physiological.

CONCLUSIONS

Cessation of breeding and castration of female mice approaching the period of declining reproductive power have at least two effects upon the subsequent life history of these animals.

1. The average expectation of life is appreciably increased.
2. The incidence of tumor is markedly decreased, especially in the older age groups.

as employed by Fisher, indicates the square of the standard deviation, *i.e.*, $V = \sigma^2$. The simple zero order correlations were calculated by the usual statistical methods, the text by Wallace and Snedecor (4) being employed as a guide. For determining the significance of these correlation coefficients, the formula

$$t = \frac{r}{\sqrt{1 - r^2}} \cdot \sqrt{n' - 2}$$

was employed. Published tables (2) are available for transferring t into probability terms. In all statistical procedures, significance has been attached to values of $t \geq 2.5$ or $P \leq 0.01$; that is, when the probability of an event occurring by chance alone was 1 or less than 1 in 100, the result was considered significant.

In a few instances the use of absolute values was abandoned in favor of relative values. This procedure was adopted in order to place the observations on an equal basis by reducing them all to percentage terms. In this way the observations on the weights of fetuses 28 days old, for instance, could be summated with those of 30 day fetuses since age as a variable was eliminated.

A description of the method employed for the graphic presentation of the observations has been presented in detail elsewhere (5). The horizontal broken lines in the figures block off intervals on the ordinate which are equal to the estimated value for the standard error of the depicted means. When the heights of any two vertical bars are separated by at least two and a half such intervals, then the difference between the represented means is significant.

RESULTS

The material on which the analysis is based is presented in Table I. There were 475 fetuses carried by the 71 does, the number per doe ranging from 1 to 12, with a modal value of 7. The number of fetuses per uterine horn varied from 0 to 7, with 4 as the modal class.

Fetal Weight and Age.—The average gestation period of the rabbit is 31 and a fraction days (6). As shown in Table II, 63 or 88.7 per cent of the 71 does were sacrificed from 28 to 31 days after the last fertile mating, and these does carried 401 or 88.2 per cent of the total of 455 fully developed fetuses. The weights of 20 degenerated fetuses were not included. The relative daily increase in mean fetal weight was as follows: from 27 to 28 days, 16.3 per cent; from 28 to 29 days, 26.4 per cent; from 29 to 30 days, 8.1 per cent; from 30 to 31 days, 6.5 per cent. These values represent an average relative daily increase of 14.3 per cent for gestation periods ranging from 27 to 31 days. It is of interest to note that as the gestation period approaches its normal limit of 31 days, the relative daily increase is progressively retarded. Whether this retardation is the result of an inherent growth potentiality of the developing fetus, or is due to an exhaustion of the nutritive and tensile capacity of the maternal uterus, is a question which cannot be answered on

were descended in pure line from original standard bred stock. Similarly, the stud bucks were for the most part derived from hybrid ancestry, although an occasional standard bred animal was represented. The rabbits in the colony are all housed in individual cages, and the diet comprises a standard uniform ration. Matings are personally supervised by members of the staff. To procure the desired mating, the doe is placed with the buck in the latter's cage, and after copulation, returned to its own cage. It is possible after the lapse of a 10 day interval to determine the pregnant or non-pregnant state of the doe by abdominal palpation. If pregnant, it is permitted to go to term; if non-pregnant, the desired mating is repeated. In this way the date of the copulation which resulted in fertilization is obtained.

An effort was made in the present investigation to examine the fetuses as near term as possible. At intervals ranging from 23 to 31 days after the fertile copulation, the pregnant does were sacrificed by air injection into the marginal ear vein. The pelvic organs were immediately exposed and the uterine horns opened. Each fetus was removed from its membranes, and the placentae carefully detached from the uterine wall. The weight of each fetus and of the corresponding placenta was then determined by means of a Toledo automatic balance calibrated in 1 gm. intervals. In addition, careful records were made of the uterine horn in which the fetus was located, that is, whether right or left, and of the fetal presentation and position or order. By presentation is meant that part, head or breech, which is directed toward the uterine outlet. The position or order indicates the relative locus of the fetus in the horn, the first position being that nearest the Fallopian tube. In summary, the following information was available with reference to each fetus: age, weight, weight of placenta, horn, presentation, and position or order.

Statistical Analysis.—In deriving the significance of the difference between the right and left uterine horns with regard to such variables as number of fetuses, total weight of fetuses, mean weight of fetuses, etc., the mean difference and its standard error were calculated. The mean difference (Md) was obtained by the formula $\frac{\Sigma d}{n}$, and its standard error from the formula

$$\sqrt{\frac{\Sigma(d)^2 - \Sigma d \cdot Md}{n(n-1)}},$$

d representing the difference between the numerical observations on the right and left horns of any doe. This method of comparison eliminates the variation due to fetal age, breed, maternal weight, and other uncontrolled variables. In a few instances comparisons were made by the use of the standard error of the mean and the standard error of the difference between means.

The χ^2 test of homogeneity is described by Fisher (2) who also gives tables for translating various levels of χ^2 into terms of probability. The method of analysis of variance in which the ratio F is used is that described by Snedecor (3), F being a symbol for the ratio of the larger to the smaller variance. The term "variance"

TABLE I—*Continued*

Doe's No. in series	Gestation period	Presentation	Horn and position	Weight	
				Fetus	Placenta
	days			*gm.*	*gm.*
11	28		L1	34	5.5
			L2	34	4
			L3	34	4
			L4	34.5	4.5
			R1	38.5	6
			R2	34	6.5
12	28	H	L1	38.5	5
		B	L2	32	4
		H	L3	28	4
			R1	D	D
		B	R2	36	5
13	27	H	L1	25	4.5
		H	L2	23.5	3.5
		B	L3	25	4
		H	L4	24	4
		B	L5	27	4
		H	R1	25.5	4.5
		H	R2	26	4.5
		B	R3	28	5
		B	R4	28	4.5
14	27	H	L1	28	4.5
		H	R1	25	4
		H	R2	21.5	4
		B	R3	21.5	3
		H	R4	26	4
		H	R5	28	4.5
15	27	H	L1	34	6
		H	L2	30	5
		H	L3	33	6
		H	L4	31	5
16	28		L1	30	5
			L2	27	4
			L3	31	5
			L4	28	5
			R1	33	5
			R2	29	5
17	28	B	L1	32	5
		H	L2	37	6.5
		B	L3	38	7
		B	L4	40	8
		B	R1	36	7
		H	R2	38	8
		H	R3	27	5
		H	R4	29	5
18	28	B	R1	35.5	10
19	28	H	L1	33	4.5
		H	L2	27	3
		H	L3	32	4
		H	L4	23	3
			L5	D	D
		B	R1	27.5	3
		H	R2	30	4
		H	R3	25	3.5
		H	R4	27	4
20	29	H	L1	38.5	4.5
		B	L2	34.5	4
		B	L3	34	4
		H	R1	42	5
		H	R2	40	4
		B	R3	38	4
		H	R4	37	4
21	27	H	L1	32	4
		H	L2	27	4
		H	L3	24.5	3.5
			L4	D	D
		H	L5	30	4
		B	L6	28	4
		B	L7	24	3
		H	R1	31	4
		H	R2	30	4
		B	R3	26.5	4

TABLE I

The Weights of Rabbit Fetuses and Corresponding Placentae

Doe's No. in series	Gestation period	Presentation	Horn and position	Weight	
				Fetus	Placenta
	days			*gm.*	*gm.*
1	27	H	L1	27.5	5
		H	L2	29	4.5
		B	R1	26	5
			R2	D	D
		B	R3	31	6
		B	R4	25	4.5
2	28	B	L1	30	6.5
		H	L2	29	4.5
		H	L3	28	4.5
		H	R1	32.5	7
3	28	H	L1	37	6
		H	L2	36	5
		H	L3	37	5
		H	L4	34	6
		H	L5	40	5.5
		B	R1	36	5.5
		B	R2	38	6
4	27	B	L1	25	4
		H	L2	32.5	6
		H	L3	27	4
		B	L4	26	4
		B	R1	28	4.5
		B	R2	29	6
		B	R3	30	5
		H	R4	28.5	4.5
5	29	H	L1	46	3.5
		H	L2	49	6
		H	R1	48	5
		B	R2	46	4
		B	R3	44	4
			R4	D	D
		H	R5	46	4.5
		B	R6	50	5.5
6	28	H	L1	35	6
		H	L2	38	6.5
		H	R1	36	5.5
7	28	H	L1	29	4.5
		B	L2	29	4.5
		H	L3	23	4
		B	L4	25	3.5
		H	L5	21	3.5
		H	L6	25	4
		B	R1	24	4
8	28	H	L1	28	4
		H	L2	27	5
		H	L3	22	5
		B	L4	25	3
		H	L5	21	3
		B	L6	23	3
		B	R1	30	5
			R2	29	4
			R3	D	D
			R4	D	D
			R5	D	D
9	28	H	L1	31	4.5
		B	L2	27	4
		B	L3	30	4
		B	L4	25	4
		H	R1	31	5
		H	R2	32	6
		B	R3	26.5	4
		H	R4	29	4.5
10	28	H	L1	40	7.5
		H	L2	37	4.5
		B	L3	36	7.5
		H	L4	33	5.5
		H	R1	31	5
		H	R2	35	7
		B	R3	30	6
		H	R4	33.5	5.5
		H	R5	28	5.5
		H	R6	33	6

H = Head; B = Breech. L1 = left horn, first position; R3 = right horn, third position, etc. D = Degenerated.

TABLE I—*Continued*

Doe's No. in series	Gestation period	Presentation	Horn and position	Weight	
				Fetus	Placenta
	days			*gm.*	*gm.*
33	26	B	L1	24	5
		H	L2	23	5.5
		H	L3	22	5
		H	L4	20	4.5
		H	L5	21	5
			R1	D	D
			R2	D	D
		B	R3	22.5	5
		H	R4	22	5
34	23	H	L1	9	3
		H	L2	10	3
		H	R1	9	3
		H	R2	8.5	2.5
		H	R3	10	3
		B	R4	10	3
35	30	B	L1	44	4
		H	L2	40	4
		B	L3	45	3
		B	R1	46	4.5
		H	R2	41	4
		B	R3	45	4.5
		B	R4	45	4
36	30	B	L1	48	4.5
		H	L2	43	4.5
		H	R1	44	4.5
		H	R2	40	4
		H	R3	39	4
		B	R4	40	4.5
37	29	H	L1	41	5
		H	L2	40	5
		H	R1	38	5
		B	R2	39	4
		H	R3	36	4
		B	R4	36	4
		H	R5	35	4
38	29	H	L1	46	6
		H	L2	47	6
		H	L3	43	5
		H	L4	33	4
		H	R1	44	5
		H	R2	42	5
		B	R3	46	5
		B	R4	39	4
39	29	H	L1	37.5	5
		B	L2	37.5	5
		B	R1	41	5
		B	R2	38.5	5.5
		B	R3	36	4
		H	R4	34	3
40	28	H	L1	33	4
		H	L2	39	5
		B	R1	35	5
		B	R2	37	6
		H	R3	36	5
41	29	B	L1	40	5.5
		B	L2	48	6.5
		B	R1	40	6
		B	R2	40	4.5
		H	R3	42	4.5
		H	R4	41	5
		B	R5	42	5.5
42	28	B	L1	36	5
		B	R1	40	6
		H	R2	38	5
		H	R3	39	6
43	31	B	L1	52	7
		B	L2	50	6.5
		B	L3	45	6
		H	R1	52	7

TABLE I—*Continued*

Doe's No. in series	Gestation period	Presentation	Horn and position	Weight		Doe's No. in series	Gestation period	Presentation	Horn and position	Weight	
				Fetus	Placenta					Fetus	Placenta
	days			*gm.*	*gm.*		*days*			*gm.*	*gm.*
22	28	B	L1	32	5	26	29	B	R2	31.5	4
		B	L2	30	5			B	R3	35.5	4.5
		H	R1	32.5	5.5			B	R4	29	4
		B	R2	30	4.5						
		H	R3	30	5	27	29	H	L1	43	4
		B	R4	26	3.5			H	L2	50	6.5
								H	L3	36	3
23	28	B	L1	31	4			H	L4	43	4
		B	L2	26	3.5			B	R1	45.5	5.8
		B	L3	25	3			H	R2	47	4
		H	L4	25.5	3				R3	D	D
		H	R1	32	5			H	R4	41	3.5
		H	R2	35	5						
		B	R3	28	3	28	30	H	L1	49.5	4
		H	R4	29	3			H	L2	49	3.5
		B	R5	21	3			B	R1	43	3
		H	R6	24	2			B	R2	50	4
		B	R7	30	4			H	R3	50	4
24	29	B	L1	36	4	29	30	H	L1	40	4
		H	L2	34	4			H	L2	28.5	3.5
		B	L3	30	3.5			B	L3	35	4
		H	L4	30	3.5			H	L4	27	3
		B	L5	34	3.5			H	L5	35	4
		B	R1	47	5.8			H	R1	36	4
		H	R2	38	4			B	R2	31.5	4
		B	R3	38.5	4			B	R3	36	3
		H	R4	38.5	4						
						30	30		L1	D	D
25	29	H	L1	41.5	5			H	L2	54	5.5
		B	L2	36	4			B	R1	50	5.5
		B	L3	41	4.5			H	R2	47	5
		B	L4	37.5	4			H	R3	50	6
		H	L5	33.5	4						
		H	R1	45	5	31	31	H	L1	49	8
		B	R2	45.5	5.5			B	L2	51	7
		H	R3	42	5.5			B	R1	48	7
26	29	H	L1	33.5	5	32	31	H	L1	51	5.5
		H	L2	32	4			H	L2	53	6
		B	L3	35	5			H	L3	43	5
		H	R1	38	5.5			B	L4	45.5	5
								B	R1	50	6

TABLE I—*Continued*

Doe's No. in series	Gestation period	Presentation	Horn and position	Weight	
				Fetus	Placenta
	days			*gm.*	*gm.*
54	29	B	L1	45	8
		B	L2	47	7
		H	R1	48	7
		H	R2	42	6
		B	R3	40	6
55	29	B	L1	40	5
		B	L2	40	5
		B	L3	43	5
		B	L4	39	5
		H	L5	39	5
		B	R1	43	7
		B	R2	38	5
56	29	B	L1	33	5
		B	L2	33	4
		B	R1	36	5
		H	R2	34	6
		H	R3	32	5
		B	R4	39	5
57	29	H	L1	40	5
		B	L2	32	5
		H	R1	47	8
		H	R2	34	6
58	31	B	L1	44	6
		H	L2	47.5	9
		B	L3	43	5
		H	R1	46	7
		H	R2	37	4.5
		H	R3	38	6
		B	R4	42	6
59	31	B	L1	54	6
		B	R1	60	6.5
			R2	D	D
			R3	D	D
		H	R4	44	4
		H	R5	51	5
60	29	H	L1	53	8
		H	L2	43	5
		B	L3	45	6
		B	L4	32	4
		B	R1	45	7
		H	R2	48	8
		B	R3	48	6
61	29	H	L1	45	5
		H	L2	50	6
		H	R1	50	7.5
		H	R2	46	6
		H	R3	39	4
62	31	H	R1	63	6
			R2	D	D
		B	R3	67	6
		H	R4	53	5
63	28	H	L1	29	4
		H	L2	30	4
		B	L3	31	6
		H	L4	33	4
		B	R1	34	4
64	30	H	L1	50	7
		H	L2	52	6
		B	L3	47	5
		H	L4	47	5
		H	R1	51	6
		B	R2	48	5
65	30	H	L1	52	6
		H	L2	47	6
		B	L3	40	4
		H	L4	33	4
		B	L5	45	5
		H	R1	50	6
		B	R2	48	7
		B	R3	47	6

TABLE I—*Continued*

Doe's No. in series	Gestation period	Presentation	Horn and position	Weight		Doe's No. in series	Gestation period	Presentation	Horn and position	Weight	
				Fetus	Placenta					Fetus	Placenta
	days			*gm.*	*gm.*		*days*			*gm.*	*gm.*
44	31	B	L1	34	5	49	29	H	R1	46	6
		B	L2	31	4			B	R2	44	6
		B	L3	38	5.5			B	R3	44	5.5
		B	L4	32	4.5			H	R4	47	5.5
		H	L5	29	4			H	R5	44	6
		B	R1	38	5.5						
						50	29	H	L1	46	6
45	29	H	L1	42	4			H	L2	45	5
		H	L2	32	4			H	L3	43	5
		H	L3	35	4			B	R1	45	6
		B	R1	37	5			H	R2	43	6
		B	R2	28	3			H	R3	41	5
		B	R3	34	5			H	R4	42	5
		H	R4	30	3						
		H	R5	35	5	51	29	H	L1	52	10
									L2	D	D
46	29	B	L1	44	7.5				L3	D	D
		B	L2	38	6			H	R1	43	7
		B	L3	38	5			B	R2	48	9
		B	R1	40	6			B	R3	47	8
		H	R2	40.5	6						
		B	R3	39	5.5	52	29	H	L1	45	7
		H	R4	40	5			B	L2	45	8
								B	L3	47	7
47	29	B	L1	38	5			H	L4	42	7
			L2	D	D			H	L5	44	6
		B	L3	36	5			H	R1	47	6
		B	L4	29	4			H	R2	40	7
		B	L5	35	4			H	R3	42	8
		H	R1	40	4.5			H	R4	44	7
		H	R2	36	4.5			H	R5	37	6
		H	R3	27	3.5			H	R6	33	4
								B	R7	36	6
48	29	H	L1	48	6						
		B	L2	39	4						
		B	L3	40	5	53	29	H	L1	43	6
		B	R1	43	5			H	L2	25	2.5
		B	R2	38	4			H	L3	33	4
								B	L4	38	4.5
49	29	B	L1	50	7			B	L5	35	4
		H	L2	43	5			B	R1	42	5
		H	L3	46	5.5			B	R2	36	4
		B	L4	43	6			H	R3	36	4

Comparison of the Number of Fetuses in Each Uterine Horn.—A total of 475 fetuses were observed. Of these, 236 were in the right horn and 239 in the left. The mean difference of 0.042 ± 0.140 was statistically insignificant. In Ibsen's (8) observations on the guinea pig there was likewise no difference between the number of fetuses in the right and left horns. 32 does of the present series had a greater number in the right than in the left horn, 31 had a greater number in the left horn, and 8 had an equal number in each horn. These values are not any different from those one would expect by the operation of chance alone. 20 or 4.2 per cent of the 475 fetuses were degenerated and non-viable. 7 of these were located in the left horn as compared with 13 in the right. ($\chi^2 = 3.6$, not significant.) The 20 degenerated fetuses were found in 15 different does; in 6 of these the degenerated individuals were located in the left horn and in 9 in the right. The difference between these values is not significant ($\chi^2 = 1.2$). In summary, the observations indicated that there was no difference between the number of fetuses in each uterine horn; that in any particular doe the chances were equal that the number of fetuses in either horn would be greater or less than the number in the other horn; that when degeneration of a fetus occurred, it had an equal chance of taking place in either horn; and that if a given doe had a degenerated fetus, there was an equal chance that it would be found in either horn.

The Frequency of Head and Breech Presentation, and the Influence of Right and Left Sidedness on Presentation.—Presentation is here taken to mean that part of the fetus which is directed toward the uterine outlet, *i.e.*, head or breech. Transverse presentations were not encountered. The presentation of 443 fetuses carried by 69 does was recorded. The head was the presenting part in 247 or 55.7 per cent of these, and the breech in 196 or 44.3 per cent. The difference between these values is statistically significant ($\chi^2 = 11.56$, $P < 0.01$), indicating that in our material, head presentation was significantly more frequent than breech. Ibsen (8) has noted a similar disproportion between head and breech presentation in the guinea pig.

There were 219 fetuses in the right uterine horn; 115 or 52.5 per cent had the head as the presenting part. Of the 224 in the left horn, 132 or 58.9 per cent were head presentation. The difference between these values is statistically not significant ($\chi^2 = 1.84$). Thus the uterine horn in which the fetus was located had no influence on its presentation.

The Relation between Presentation and Position.—The order of the fetus in the uterine horn, whether first, second, third, etc., has been denominated "position." The first position is that highest in the uterine horn, nearest the Fallopian tube. Table III presents the relation between the presentation and position of the fetuses. An analysis of these values indicates that the ratio between the frequency of head and breech presentations in the several positions is probably significantly different from the ratio represented in the totals ($\chi^2 = 7.95$, $P = 0.05$). It is evident that the principal discrepancy exists in the third position, in which there is an excess of fetuses with breech presentation. The reason for this discrepancy is not at present understood.

Comparison of the Total and Mean Fetal Weight in Each Horn.—Considering only

TABLE I—*Concluded*

Doe's No. in series	Gestation period	Presentation	Horn and position	Weight		Doe's No. in series	Gestation period	Presentation	Horn and position	Weight	
				Fetus	Placenta					Fetus	Placenta
	days			*gm.*	*gm.*		*days*			*gm.*	*gm.*
66	29	H	L1	37	5	69	29	H	L1	38	6
		B	L2	35	4			H	L2	34	5
		B	L3	36	4			H	L3	28	5
		B	L4	35	4			H	L4	28	5
		H	L5	42	5			H	L5	31	4
		H	R1	40	7			B	R1	43	8
		H	R2	38	4				R2	D	D
						70	28	H	L1	35	7
67	28	B	L1	34	4			B	L2	35	7
			L2	D	D			H	L3	34	6
		B	L3	35	5			B	R1	46	6.5
		H	R1	31	4.5			B	R2	33	7
		B	R2	36	4.5			H	R3	35	6
		B	R3	37	5			B	R4	27	5
								B	R5	32	6.5
68	29	H	L1	40	5	71	28	B	L1	37	5
		H	L2	38	4.5			H	L2	35	5
		H	L3	36	4			H	R1	26	5.5
		B	L4	30	3			H	R2	30	5
		B	R1	40	6			B	R3	30	5
		H	R2	36	5			H	R4	30	5
		B	R3	34	3			B	R5	33	5.5

TABLE II

*The Relation between Fetal Weight and Age**

Age, *days*	23	26	27	28	29	30	31
No. of does	1	1	6	21	28	7	7
No. of fetuses	6	7	41	131	194	44	32
Mean weight of fetuses, *gm.*	9.42	21.6	27.0	31.4	39.7	42.9	45.8
Standard deviation of mean, *gm.*	—	—	0.45	0.42	0.40	0.79	1.62

* The weights of 20 degenerated fetuses are not included.

the basis of the present observations. Draper (7) has noted a similar retardation in the fetal growth of the guinea pig. In this species, fetal growth occurs rapidly from the 15th to the 25th day, and thereafter the relative growth rate decreases rapidly at first and then more slowly for the rest of the gestation period.

The Influence of Presentation on Fetal Weights.—To determine whether there existed any relationship between fetal presentation and weight, two methods of analysis were employed. The first was based on the fetal weights in litters containing at least two fetuses with each variety of presentation. There were 51 such litters; for each of these, the average weight was calculated for those individuals with head and for those with breech presentation. The average weight of head presenting fetuses was greater than the average for breech presenting fetuses in 20 litters; the opposite was the case in 28 litters, and the average weights for head and for breech individuals were the same in 3 litters. There is no significant difference between these values ($\chi^2 = 2.68$). The mean difference between the averages for head and breech fetuses also was statistically insignificant although the weight of breech was slightly less than that of head fetuses (Mean difference $= 0.164 \pm 0.24$ gm.).

TABLE IV

The Relation between Fetal Weight and Order of Implantation in Uterine Horn Combined Right and Left Horns*

Position or order	1	2	3	4 and 4+	Total
No. of fetuses	89	87	88	95	359
Mean weight, *per cent*	105.99	99.89	98.12	95.81	99.88

Analysis of Variance

Source of variation	Degrees of freedom	Sum of squares	Mean square
Total	358	57,320.9	
Between means of position classes	3	5,483.8	160.1
Within position classes	355	51,837.1	1827.9
			146.0

* Fetal weight is expressed as a ratio to the mean fetal weight in the corresponding horn. Only those horns containing 3 or more fetuses are included.

Since by the above method the average weight was in some cases determined by only two values, and since such an average was less accurate than others based on four or five values, an alternate method of analysis was employed. There were 29 does in which a head and a breech presenting fetus were found in the first position. In this group there were 18 fetuses with breech presentation in the left horn as compared with 11 in the right. There is no significant difference between these values, indicating that with respect to the distribution of head and breech fetuses in each horn the series is homogeneous. In 16 of these 29 pairs the weight of the head presenting fetus was greater than that of its breech presenting mate; in 11 the opposite was observed, and in 2 the weights of both were similar. These values

the 56 does with fetuses which were all normally developed and showed no evidence of degeneration, the total fetal weight in one horn as compared with the other was not significantly different although the weight in the left horn was slightly greater than that in the right (Mean difference = 5.83 ± 4.73 gm.). The total fetal weight was greater in the left than in the right uterine horn in 26 does, and in 30 does the total fetal weight in the right horn was greater than in the left. The difference between these findings is not significant ($\chi^2 = 0.56$). Since it has already been seen that the total number of fetuses in each uterine horn was essentially the same, and since the horns showed no difference with regard to the total fetal weight in each, one would expect that the mean fetal weight in each horn should be similar. This actually was the case. Of 28 does bearing three or more fetuses in each horn,

TABLE III

*The Relation between Fetal Presentation and Position**

Presentation	Position								Total
	1		2		3		4 and over		
	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	
Head	76	73.8	70	64.1	40	51.9	61	57.4	247
Breech	56	58.2	45	50.9	53	41.1	42	45.6	196
Total	132	132	115	115	93	93	103	103	443
x	2.2		5.9		11.9		3.6		
$S\frac{x^2}{m}$	0.15		1.12		6.18		0.50		7.95

x = difference between observed and expected value. m = expected value. S = sum.

* 12 fetuses of 2 does were omitted because their presentation was not recorded.

the mean difference between the average fetal weights in the right and left horns was 0.73 ± 0.41 gm., a statistically insignificant value. In 16 of these does the mean fetal weight in the right horn was greater than the mean fetal weight in the left; in 10 the reverse was found, and in 2 the mean weight in each horn was the same. These values are not any different from those one would expect by the operation of chance alone ($\chi^2 = 2.76$). To summarize, there was no difference between the horns with regard to total fetal weight; in any doe, the chances were equal that the total fetal weight in one horn would be greater or less than the total fetal weight in the other; in a group of does each bearing three or more fetuses in each horn, the average fetal weights in one horn were no different from the average fetal weights in the other; and the chances were equal that in any doe bearing three or more fetuses in each horn, the mean fetal weight in one horn would be greater or less than the mean fetal weight in the other.

the weight of the fetus is significantly influenced by its position in the uterine horn. Considering only the first 33 does in the series, there were 54 uterine horns containing two or more fully developed fetuses. In three of these horns the first and last position fetuses had identical weights. However, 35 or 68.6 per cent of the remaining 51 first position fetuses were heavier than their mates occupying the last position in the corresponding horn, and this is significantly greater than would be expected by random sampling alone ($\chi^2 = 14.16, P < 0.01$, significant). In terms of absolute weight values, the mean difference between the weights of the first and last fetus in the left horn was $+2.2 \pm 0.51$ gm. ($t = 4.3, P < 0.01$, significant), and in the right horn $+1.92 \pm 0.52$ gm. ($t = 3.7, P < 0.01$, significant).

The above observations indicated the desirability of a more precise analysis of the relation between position and fetal weight. This is presented in Table IV and Text-fig. 1. Each uterine horn of the 71 does was considered individually, and those containing fewer than three fetuses were excluded. The fourth and succeeding fetuses were classified into one group, and the weights of each individual were expressed as a ratio to the mean fetal weight in the respective horn. The employment of relative instead of absolute fetal weight values eliminates any variability which might be due to fetal age, maternal weight, breed, etc., and places all observations on a comparable basis. It is seen that there is a progressive decrease in mean fetal weight from a high value of 106.0 per cent in the first position to a low of 95.8 per cent in the fourth and over position. By the method of analysis of variance it was found that the variance between the mean values for each position was significantly greater than the variance of the values within the position classes ($F = 12.51$, significant). This demonstration of heterogeneity between position classes indicates that the weight of the fetus is significantly influenced by position.

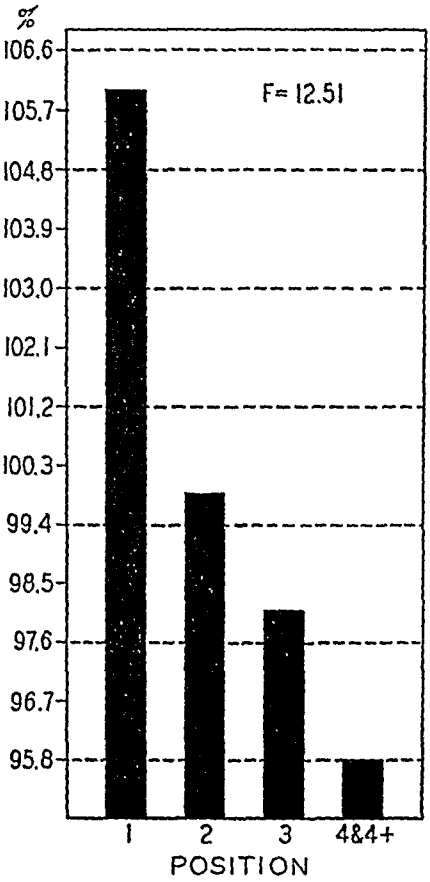
Since the above analysis considered only those uterine horns containing three or more fetuses, it does not indicate the influence of position on fetal weight when there are only two or only three or only four fetuses in a horn. A further analysis was undertaken with this consideration in mind.

1. Two Fetuses in a Horn.—25 uterine horns of 24 does contained only two fetuses each. 14 of these, occupying the first position had weights greater, and 9 had weights less than their mates in the same horn. In two horns both fetuses had identical weights. There is no significant difference between these values ($\chi^2 = 2.50$). The mean difference between the weights of the first and second position fetus was $+0.74 \pm 0.60$ gm., an insignificant difference. Thus, when there were only two fetuses in any uterine horn, position did not materially influence their weights.

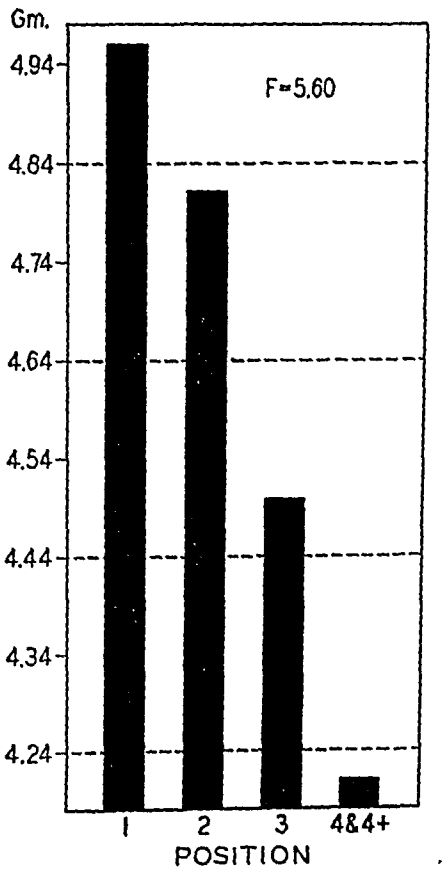
2. Three Fetuses in a Horn.—There were 28 uterine horns of as many does in each of which three fetuses were found. Here again, by employing relative weights obtained from the ratio of the absolute weight of a fetus to the mean fetal weight in the horn, there was noted a progressive decrease in weight with increasing distance from the Fallopian tube. The mean relative weight in position 1 was 104.37 per cent, in position 2, 98.87 per cent, and in position 3, 96.88 per cent.

do not differ from chance distribution ($\chi^2 = 0.64$). The mean difference, 0.40 ± 0.51 gm., between the weights of breech and head fetuses is not significant, but the mean weight of the breech fetuses was slightly greater than that of the head fetuses. The analysis thus indicates that fetal weight was not influenced by presentation.

The Influence of Position on Fetal Weight.—A preliminary survey indicated that



TEXT-FIG. 1



TEXT-FIG. 2

TEXT-FIG. 1. The relation between fetal weight and position.

TEXT-FIG. 2. The relation between placental weight and position.

Fetal weight is expressed as a ratio to the average weight in the corresponding horn. In each case the horizontal dotted lines intersect intervals on the ordinate equal to the estimated value for the standard error of the difference between any two means. When the heights of any two vertical bars are separated by at least two and a half such intervals, the populations represented are significantly different.

heaviest fetus weighed more than the placenta of the lightest fetus, and the difference varied from 0.5 to 3.0 gm. There thus appeared to be a high positive correlation between fetal and placental weights. The character of this correlation was investigated further by the method of linear correlation. The coefficient of correlation between fetal weight and placental weight was $+0.3521$ ($n = 161$, $t = 4.7$, $P < 0.01$), which is highly significant. In determining this coefficient, the 161 observations on the first 28 litters in the series were employed, and fetal weight was expressed as a percentage of the mean weight of the corresponding litter. Absolute values for placental weight were employed however. Had placental weight been placed on the same relative basis as fetal weight, a still higher correlation coefficient would have been noted. This statement is based on a comparative analysis of the 57 fetuses and placentae of the first 9 does. When both the fetus and the placenta were given a relative value based on the ratio to the mean weight in the doe, the coefficient of correlation was $+0.4582$ ($n = 57$, $t = 3.8$, $P < 0.01$), while when relative values were employed for the fetus only and absolute values for the placenta, the smaller coefficient of $+0.3378$ ($n = 57$, $t = 2.6$, $P < 0.01$) was obtained.

This finding of a high positive correlation between fetal and placental weight merely indicates that in general heavy fetuses and heavy placentae, and light fetuses and light placentae are associated. From these observations one would expect that placental weight should be influenced by the order of implantation in the uterine horn in exactly the same manner that fetal weight was so influenced. This in fact was found to be the case. Considering only the first 33 does, and grouping the observations without regard to uterine horn, the following results were obtained: 54 placentae in position 1 had a mean weight of 4.96 ± 0.133 gm.; 55 placentae in position 2 had a mean weight of 4.81 ± 0.149 gm.; 44 placentae in position 3 had a mean weight of 4.50 ± 0.155 gm.; and 55 placentae in position 4 and over had a mean weight of 4.21 ± 0.136 gm. (Text-fig. 2). The variance between the means of classes was significantly greater than the variance within classes ($F = 5.60$), indicating significant heterogeneity between each position.

In the final analysis the explanation for the decreasing weight of

The variance between the means for each position was significantly greater than the variance of the values comprising each position class ($F = 11.17$, significant). From this it follows that when there were three fetuses in a horn, position significantly influenced fetal weight, fetuses in the first position in general weighing most, those in the third position weighing least, and those in the second position having an intermediate weight.

3. Four Fetuses in a Horn.—29 does had 34 uterine horns containing four fetuses each. The weight of each fetus was again expressed as a ratio to the mean fetal weight in the horn. The mean ratio for each position ranged from a high value of 105.55 per cent in the first position, to a low value of 94.83 per cent in the fourth position, with intermediate values of 101.04 per cent in the second and 98.38 per cent in the third positions. There was significant heterogeneity between each position as indicated by the significant value of $F = 13.31$. It is evident that when there were only four fetuses in a horn (and it will be recalled that four represented the modal class for number of fetuses per horn), their weights were significantly influenced by their position in the uterine horn, those located highest in the horn having in general the greatest weights, those lowest in the horn near the outlet having the lowest weights, and those occupying intermediate positions having intermediate weights. Ibsen (8) noted in the guinea pig that in the 65 day stage those fetuses nearest the ovaries weighed approximately the same as those nearest the vagina. During the later stages, however, the fetuses nearest the ovary and the vagina averaged more in weight than those between these extremes.

DISCUSSION

Of major interest in the foregoing analysis was the observation that fetal weight at or near term was significantly influenced by the position or order in the uterine horn. In general, the weights of fetuses implanted high up in the horn nearest the Fallopian tube were greater than those developing nearest the outlet, and fetuses occupying intermediate positions had intermediate weights. In attempting to arrive at an explanation for this phenomenon, our attention was directed to the placenta.

A preliminary analysis indicated that there was a high degree of relationship between fetal weight and placental weight. The weights of the heaviest and lightest fetus and of the corresponding placentae were recorded for each of the first 32 litters. The mean difference between the weights of the placentae corresponding to the heaviest fetus and the weights of the placentae corresponding to the lightest fetus was $+1.37 \pm 0.128$ gm., a highly significant value ($t = 10.7$, $P < 0.01$). Moreover, in 30 of the 32 litters the placenta of the

newborn. The weaklings are frequently overpowered by their stronger sibs in the struggle for nourishment and, moreover, the doe sometimes disregards her weaker offspring and refuses to nurse them. As a result, the recorded weights may deviate from the actual birth weights in a negative or positive direction according to the relative strength or weakness of the animals comprising the litter. In order to determine to what extent these factors might account for the occurrence of dwarfs, an analysis was made of two large groups of rabbits. The first group consisted of the individuals in normal non-dwarf containing litters, and the second comprised the members of litters each containing at least one dwarf. It was concluded that when the birth weight of a rabbit as determined by the routine procedure described above is less than 50.1 per cent of the weight of its

TABLE V

Frequency Distribution of Weights of Three Groups of Rabbits Weight of Each Individual Expressed as a Percentage of the Weight of the Heaviest Litter Mate

Group	Description	100.0–85.1%	85.0–70.1%	70.0–50.1%	50.0–20.1%	Total
I	Fetal weight—normal litters	312	123	19	0	454
II	Birth weight—normal litters	378	146	37	4	565
III	Birth weight—dwarf litters	252	77	19	140	488

heaviest sib, a diagnosis of dwarfism is warranted. Since the birth weights of the unusually small, non-viable individuals all fell below the 50.1 per cent class, they were regarded as true dwarfs in the sense that their abnormally low weights could not be ascribed to environmental and nutritive factors operative in the interval between birth and the weight determination.

It will be recalled that the present observations were initiated for the particular purpose of ascertaining to what extent intrauterine factors could account for these dwarf individuals. The quantity of nutriment obtained by the fetus is independent of its own efforts, so that in a consideration of fetal weight at or near term one element which might account for variability in birth weight, namely, nursing, is absent. Table V presents a comparison of the weights of three

fetus and of placenta with increasing distance from the Fallopian tube cannot be found in the present data. Two hypotheses, however, are suggested. The first is that fetal weight is determined in a large measure by placental mass, and that placentae high up in the horn are larger than those lower down because of the differential in the vascularity and nutritive efficiency of the uterus in these locations. Whether this gradient of vascularity actually exists, however, is not known. The second hypothesis is that fetal and placental weights are correlated with each other, and that variability in both is caused by a third factor, namely age. On this basis, fetuses and their corresponding placentae which are located nearest to the Fallopian tube are older than those developing lower down in the uterine horn and, therefore, weigh more. This hypothesis suggests that there is a time differential in the maturation, discharge and fertilization of the ova, that the first ovum to be fertilized is in general implanted in that portion of the horn nearest the ovary, and that the last ovum to be fertilized is implanted nearest the uterine outlet. The test of this hypothesis must await further investigation. It should be stated, however, that Walton and Hammond (9), in experiments involving direct observation of the maturing ovarian follicle of the rabbit, noted that the follicles of the same batch do not rupture at exactly the same time, for in several cases where observations began about 10 hours after coitus, one or more follicles had already ruptured, while others ruptured subsequently under observation.

An earlier communication (10) has reported the criterion for the diagnosis of dwarfism on the basis of weight at birth. Birth weights have been obtained in this laboratory by the following procedure. Each morning rabbits born during the previous 24 hour period are identified by color markings or toe markings and weighed to the nearest gram on a Toledo automatic balance calibrated in 1 gm. intervals. The average elapsed time from birth to weighing is 12 hours, the midpoint of the 24 hour interval between determinations. It was apparent that this routine introduces an error which is dependent on the amount of nourishment which each individual in the litter has obtained between birth and the weight determination. This error varies with the length of the intervening period and also with the number in the litter and the relative strength or weakness of the

There was no significant difference between the number of fetuses in each uterine horn.

Head presentation was significantly more frequent than breech, but the uterine horn in which the fetus was located had no influence on its presentation.

A greater relative number of breech presenting fetuses was observed in the third position than in the other positions.

Presentation did not exert a significant influence on fetal weight.

Fetal weight at or near term was significantly influenced by the position or order in the uterine horn. In general, the weights of fetuses implanted high up nearest the ovary were greater than those developing nearest the outlet, and fetuses occupying intermediate positions had intermediate weights. When, however, only two fetuses were present in a horn, position had no effect on their weights.

A significant positive coefficient of correlation was observed between fetal and placental weights. Moreover, placental weight was influenced by position in the uterine horn in exactly the same manner that fetal weight was so influenced.

The factors which produced variability in fetal weight at or near term, did not account for the abnormally low birth weights of the dwarf rabbits observed in this laboratory.

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groups of individuals. Group I comprises the fetal weights recorded in the present study; group II consists of the birth weights of rabbits in normal non-dwarf containing litters; and group III includes the birth weights of individuals in litters each containing at least one dwarf. In each case the weight of each individual was expressed as a ratio to the weight of its heaviest sib. The material in groups II and III has been presented elsewhere (10) in slightly different form.

An analysis of these observations by the χ^2 test of homogeneity indicates that there is no significant difference between groups I and II ($\chi^2 = 5.67$), and that both of these differ significantly from group III (I and III, $\chi^2 = 155.87$; II and III, $\chi^2 = 176.16$). Of particular importance is the fact that in no case was the weight of the smallest fetus in a litter less than 50.1 per cent of that of its heaviest litter mate, while the birth weights of all of the dwarfs fell below the 50.1 per cent class. These findings show conclusively, first, that those factors which produce variability in fetal weight at or near term cannot account for the abnormally low weights of dwarf individuals and, second, that the error introduced by our method of obtaining birth weights does not appreciably affect the relative variability in the weights of rabbits at the precise moment of birth. It is to be expected, however, that the weight of dwarf individuals would be affected by the position occupied by such individuals in the uterus.

SUMMARY

Observations were made on 475 fetuses carried by 71 pregnant rabbits. 63 or 88.7 per cent of the 71 does were sacrificed from 28 to 31 days after the last fertile mating, and these does bore 401 or 88.2 per cent of the total of 455 fully developed fetuses. The following information was available with reference to each fetus: age, weight, weight of corresponding placenta, horn, *i.e.*, right or left, presentation, and position or order. The presentation indicated that part, head or breech, which was directed toward the vagina, and position or order, the relative locus of the fetus in the horn, the first position being that nearest the ovary.

As the gestation period approached its normal limit of 31 days, the relative daily increase in mean fetal weight was progressively retarded.

There was no significant difference between the number of fetuses in each uterine horn.

Head presentation was significantly more frequent than breech, but the uterine horn in which the fetus was located had no influence on its presentation.

A greater relative number of breech presenting fetuses was observed in the third position than in the other positions.

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As the gestation period approached its normal limit of 31 days, the relative daily increase in mean fetal weight was progressively retarded.

ON THE MECHANISM OF IMMUNITY IN TUBERCULOSIS

THE HOST-PARASITE RELATIONSHIP UNDER THE CONDITIONS OF A LOCALIZED AGAR FOCUS OF INFECTION AND THE GENERALIZATION OF THE DISEASE IN NORMAL AND IMMUNIZED RABBITS*

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PLATES 52 TO 54

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There is still wide divergence of opinion as to the mechanism of immunity in tuberculosis. The extensive studies of Rich and his associates (1) on the rôle of inflammation in immunity to reinfection have cast much doubt upon the hitherto widely accepted view that allergy, which accompanies immunity in tuberculosis, is the essential mechanism thereof. On the other hand, the fundamental observations of Opie (2) on the fixation of foreign protein in the Arthus phenomenon and the illuminating experiments of Menkin (3) on the rôle of fibrin and thrombosed lymphatics in the fixation of a variety of substances, including bacteria, at the site of inflammation, would indicate that the increased inflammation incident upon reinfection may play a rôle in immunity to tuberculosis. It is almost universally accepted that the rôle of humoral substances in immunity to tuberculosis has not been demonstrated; hence immunity in tuberculosis is generally considered as entirely cellular in nature. The present investigation represents an endeavor to throw more light upon these questions.

In previous studies (4) it was shown that immunity to intravenous reinfection is a function of the increased capacity of the mononuclear phagocytes to digest tubercle bacilli. With the persistence of an

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The number of colonies cultured from known amounts of a given tissue was correlated with the histological changes in the immediately adjacent tissue. The sections were stained with hematoxylin and eosin and by the Ziehl-Neelsen method for tubercle bacilli. The Gram and fibrin stains were used in some instances.

Two types of rabbits were used in these experiments. Some had received repeated intravenous inoculations of B C G. Others had been previously inoculated with virulent bovine tubercle bacilli of the Ravenel strain. The host-parasite relationships in these two groups were compared to those in a group of normal rabbits simultaneously inoculated with the same agar-tubercle bacillus suspension.

TABLE I

The Fate of Virulent Bovine Tubercle Bacilli in a Localized Agar Focus and Their Dissemination in the Body of Normal and BCG Vaccinated Rabbits, as Indicated by the Number Found (Series 1)

Agar suspension	Interval after inoculation	Rabbit No.		Agar focus		Draining axillary nodes		Spleen		Lung	
		Normal	Vaccinated	Normal	Vaccinated	Normal	Vaccinated	Normal	Vaccinated	Normal	Vaccinated
4,450	1 day	26-2	12-0	—	3,700	0	0*	0	0	0	0
	3 days	26-5	12-5	18,060	8,900	18	1	0	0	0	0
	1 wk.	26-8	12-3	27,800	2,650	176	19	1	3	1	0
	2 wks.	26-7	12-1	40,000	5,300	10,400	73	100	1	10	0
	4 wks.	26-4	12-7	12,360	1,960	9,360	164	30	1	60	0
	80 days	26-9	12-4	—	6,880	7,200	52	13	—	11,500	14†

* Control axillary node 1.

† Adjusted from colonies obtained after treatment.

The Host-Parasite Relationships in Normal and BCG Vaccinated Rabbits

Fate of the Bacilli.—A series of rabbits received two intravenous injections of 1.0 mg. living B C G at an interval of 132 days. 20 days after the last injection the rabbits showed a marked degree of hypersensitivity to the intracutaneous administration of tuberculin. 43 days later these vaccinated rabbits, together with a group of normal animals, received a trypan blue suspension of virulent tubercle bacilli in agar subcutaneously over the right chest wall. The number of colonies isolated from 10 mg. of the agar inoculum, the subcutaneous agar focus, the draining lymph nodes, the spleen and lung of the normal and vaccinated rabbits is recorded in Table I.

It will be noted that the bacilli multiplied unhindered in the agar focus of the normal animal to the end of the 2nd week. In the vaccinated animals on the other hand there was scarcely any multiplication in this location. With this

extensive primary lesion, the bacilli of reinfection are completely destroyed and only inconspicuous nodules of mononuclear phagocytes develop wherever the bacilli had lodged, which soon disappear. If the primary lesion is largely healed, the immediate inflammatory reaction to reinfection is much more intense; accelerated formation of epithelioid and giant cell tubercles occurs associated with a less rapid destruction of the bacilli and a less rapid resolution of the lesion. This would indicate that the greatest degree of immunity is associated not with an increased but a decreased intensity of the initial inflammatory reaction. However intravenous inoculation does not correspond with natural infection. It was decided therefore to study the host-parasite relationships when the bacilli were introduced into the body in a localized area. After many trials, tubercle bacilli were finally incorporated in melted agar (5) and injected subcutaneously. The host-parasite relationships were studied as heretofore by correlating the fate of the bacilli as indicated by culture with the histological changes in adjacent tissue. This procedure affords certain advantages offered by the tissue culture method of approach and yet all the events occur within the body (6).

Methods and Materials

Sterile 6 per cent agar in saline at pH 7.4 was melted and when cooled to approximately 50°C. was intimately mixed with virulent bovine tubercle bacilli of the Ravenel strain suspended in a 1:100 dilution of Higgins' waterproof India ink in saline, or in a 1 per cent saline solution of trypan blue. The ink and the trypan blue served not only as a guide to the distribution of the bacilli within the highly viscous melted agar but also for the purpose of indicating the dissemination of these materials to the tributary lymph nodes.

The inoculation was executed as follows: To accurately measured amounts of melted agar was added a constant amount of the suspension of the bacilli. 5 cc. of the mixture was injected subcutaneously into each of the normal and immunized rabbits of a given series. One tube containing the identical mixture was reserved for culture in order to determine the number of living bacilli present in a given weight of the inoculum. Simultaneously, these rabbits received subcutaneously on the opposite side a mixture of melted agar and dye without bacilli. At varying intervals of time following inoculation a normal and a treated rabbit were killed. The number of living tubercle bacilli present in a given weight of the agar coagulum and its surrounding capsule, in the lymph nodes draining this focus, and in the internal organs, was determined by planting weighed amounts upon Löwenstein's egg medium supplemented with bone marrow infusion as previously described (7).

It will be noted that approximately twice as many tubercle bacilli were present in the original agar inoculum used for this series as in that of the previous experiment. There was no significant difference in the number of bacilli cultured 1 day after inoculation from the agar focus of the normal and vaccinated rabbits. The subsequent findings as concerns the microorganism in the agar focus of the two groups of this series were essentially the same as in the previous experiment, namely, a marked if not complete inhibition to the multiplication of the bacilli within the agar focus was apparent in the vaccinated animal, as compared with unhindered and tremendous accumulation of the bacilli in the normal animal. However, contrary to all expectations, while no tubercle bacilli had reached the draining lymph nodes of the normal animal 1 day after inoculation, 30 colonies were cultured from the superficial axillary node draining the agar focus of the vaccinated rabbit. The superficial axillary nodes on the opposite side examined at the same time contained no tubercle bacilli. It follows that instead of a retardation to the dissemination of the bacilli from the portal of entry to the draining lymph nodes there was an actual acceleration of the spread. This accelerated spread to the immediately draining lymph nodes, however, did not prevent the marked inhibition to their multiplication in the vaccinated animals, as was noted in the previous experiment. It is plain that the increased resistance to reinfection, as noted in this series, did not depend upon the fixation of the bacilli at the portal of entry but upon forces inhibiting their multiplication after their enhanced dissemination to the draining nodes.

As in the previous experiment BCG vaccination had retarded the multiplication of the bacilli at the portal of entry and in the immediately draining lymph nodes. It is noteworthy that while the deeper draining axillary lymph nodes remained practically sterile, some bacilli either passed this barrier or entered the circulation more directly, and the few that reached the lung slowly increased in number.

What now are the host-parasite relationships associated with these observations?

The Response of the Host

The Agar Focus in Normal Rabbits.— *1 day* after inoculation of agar and tubercle bacilli into a normal rabbit, there was an extensive and widespread edema and polymorphonuclear infiltration of the

quantity of inoculum, containing 4,450 bacilli, no microorganisms could be cultured from the lymph nodes draining the agar focus of either the normal or the vaccinated animals 1 day after inoculation. Thereafter bacilli accumulated in increasingly larger numbers in the axillary lymph nodes of the normal animal, and a very much more retarded though continuous increase of the microorganism took place in the nodes of the vaccinated animal to the end of the 4th week. 80 days after inoculation the bacilli persisted in practically undiminished quantity in the draining lymph nodes and accumulated in large numbers in the lung of the normal animal. In the vaccinated animals on the other hand they persisted in small numbers in the draining lymph nodes and accumulated very slowly in the lung.

TABLE II

The Fate of Virulent Bovine Tubercle Bacilli in a Localized Agar Focus and Their Dissemination in the Body of Normal and BCG Vaccinated Rabbits (Series 2)

Agar suspension	Interval after inoculation	Rabbit No.		Agar focus		Superficial axillary node		Deep axillary node		Spleen		Lung	
		Normal	Vaccinated	Normal	Vaccinated	Normal	Vaccinated	Normal	Vaccinated	Normal	Vaccinated	Normal	Vaccinated
8,400	1 days	3	13-9	9,000	11,880	0	30*	—	—	0	0	0	0
	3 days	4	13-7	17,200	9,860	5	37	—	4	1	0	0	0
	1 wk.	2	13-0	131,000	29,640	1,850	3	190	0	19	1	21	1
	2 wks.	5	12-2	492,000	3,000	32,500	150	100	20	164	2	22	3
	5 wks.	1	13-1	1,044,000	5,700	31,900	3,430	35,700	0	840	1	6,000	96

* Control axillary node 0.

It is evident that vaccination with living B C G affords a pronounced protection against a virulent infection. While the bacilli are not destroyed at the portal of entry, they are markedly inhibited in their multiplication in this location. The bacilli that reach the draining nodes and internal organs are similarly affected.

In Table II is recorded a similar experiment with the exception that the vaccinated rabbits received three intravenous injections of 1.0 mg. of B C G, the intervals being 132 and 80 days. The virulent reinfection followed 81 days after the last B C G inoculation. 1 cc. of India ink in a 1:100 dilution in saline containing the virulent bovine bacilli was mixed with 9 cc. of agar; 5 cc. of this mixture was injected subcutaneously in the same location as in the previous experiment, into the vaccinated and a normal series of rabbits.

of distinct parallel rods, like bundles of cigars, were found both extra- and intracellularly. These, as well as dispersed individual rods, were also found lying free in the agar at a distance from the cells in the fibrin threads and exudate cells between the agar islands. They were deeply acid-fast, long rods with bulbous ends, and, as noted by culture, were actively growing.

2 weeks after inoculation nodules of young epithelioid cells infiltrated with polymorphonuclears and swarming with individual bacilli were found throughout the capsule and down to the very margin of the wall (Fig. 6). About these and about the blood vessels dense accumulation of mononuclears with hyperchromatic nuclei and scanty basophilic cytoplasm with numerous mitotic figures were seen. As noted above, together with these specific changes in response to the tubercle bacilli, there were foci of foreign body giant cells, granulation and fibrous tissue due to the agar in the focus. The zone of polymorphonuclears noted above had undergone complete necrosis. Here the bacilli were present in tremendous numbers forming large skeins and loose actively growing colonies; these were much larger and more numerous, and extended far more deeply in the acellular agar islands than in the previous interval. Vast numbers of discrete individual rods swarmed in the necrotic exudate separating the agar islands (Fig. 2). The colonies and the dispersed bacilli were much less prominent in the central regions of the agar mass, although small colonies and some dispersed bacilli were found throughout the agar focus in the acellular agar islands and especially in the fibrin shreds separating these.

In this series as well as in those to be reported later, the bacilli grew in the agar adjacent to the muscle layer to a much greater extent than in that portion of the agar which lay nearest the skin. This distinctive distribution of the bacilli was invariably associated with a much greater vascularity of the cellular wall in the former region. Therefore, the nonspecific foreign body reaction to the agar was always more pronounced in the wall surrounding the agar beneath the skin, whereas the proliferation of tuberculous tissue was more pronounced in that portion of the capsule which was adjacent to the muscle layer.

5 weeks after inoculation the capsule was a vast mass of epithelioid cells with central foci of caseation. Discrete tubercle bacilli were present in considerable numbers in these tubercles, especially in those undergoing caseation. Nests of new mononuclear cell formation with mitotic figures were still present. These specific changes were interrupted here and there by foreign body giant cells, granulation tissue and more mature connective tissue. Numerous macrophages, some aggregated into nodules and containing vast numbers of individual bacilli, apparently in active growth, as judged by their morphology, occurred at the very periphery of the capsule. The center of the lesion was undergoing softening, and individual well stained microorganisms were present in great numbers. The pleomorphism of the microorganism accorded with the continued multiplication of the bacilli as observed culturally.

The Agar Focus in Vaccinated Rabbits.—

In contrast with the changes noted in normal animals, rabbits vaccinated with

tissues surrounding the agar deposit. The blood vessels in its proximity were dilated and severely congested with a slight infiltration of mononuclears in their immediate vicinity. The agar itself was broken up into fine shreds with fluid and coagulated fibrillar strands between them. The fluid of the exudate penetrated the agar islands, which was indicated by a fine uniformly distributed eosinophilic precipitate. Well preserved polymorphonuclear and red blood cells infiltrated the spaces between the islands (Fig. 1). The carbon particles were uniformly dispersed in the agar islands as well as aggregated in loose clumps. The fragments of agar were smaller and the cellular infiltration more intense at the periphery than in the deeper regions of the agar focus. In the center no cells were present in the spaces between the large agar islands. Short granular tubercle bacilli were occasionally found throughout the agar mass. At times aggregations of polymorphonuclears with ingested bacilli were found.

On the *3rd day*, the accumulation of mononuclears about the blood vessels in proximity to the agar focus was pronounced, with an occasional mitotic figure among them. Sprouting capillaries began to extend into the agar mass from these vessels. The periphery of the focus was a dense fibrin network in the meshes of which the agar was contained. The contraction of the fibrin had apparently caused the fusion of many agar particles, as indicated by the enmeshed leukocytes in the center of some of these. Polymorphonuclears and mononuclears infiltrated the fibrin zone and penetrated along the fibrin threads for a considerable distance into the agar mass. Some of the mononuclears had phagocyted agar, carbon particles, tubercle bacilli or injured polymorphonuclears. Beyond this zone the cellular infiltration and the fibrin deposits were progressively diminished. Here necrotic polymorphonuclears predominated with occasional bacilli within them. The bacilli were also seen singly or in small groups lying free in the agar or adherent to fibrin threads. In general they were definitely rod-shaped with bulbous ends; granular, coccoid and transition forms were also seen. This appearance was associated with a multiplication of the bacilli, as disclosed by culture.

On the *7th day* the capsule surrounding the agar focus had greatly thickened by a massive accumulation of mononuclears intermixed with large numbers of granulocytes. Here and there were zones of macrophages, foreign body giant cells and syncytia engulfing and surrounding agar particles. Fibroblasts and granulation tissue were prominent in some sections of the wall. Tubercle bacilli in large numbers were often present along with agar globules in the macrophages and giant cells. Abutting against the main agar mass the dense fibrin was infiltrated by a vast accumulation of polymorphonuclears, many of which were necrotic. The penetration of the agar by the fibrin and various exudate cells was more widespread and pervasive than in the previous interval. The bacilli were present in particularly large numbers in the polymorphonuclear zone, lying free in the bits of agar between them. They were found, lying free in the agar at a considerable distance from any of the cells, as prominent colonies with rods radiating in all directions from the central mass of microorganisms. Bacilli arranged in packets

2 weeks after inoculation the difference between the normal and vaccinated animals was most pronounced. Whereas in the normal rabbit the proliferation of tuberculous tissue and the massive multiplication of the bacilli both intracellularly and free in the agar were conspicuous, in the vaccinated animal the foreign body reaction to the agar with the formation of foreign body giant cells and fibrous tissue was most prominent (Fig. 7). Associated with this there was not only no further multiplication of the bacilli in the vaccinated animal, but a marked reduction in their numbers had taken place. Whatever bacilli had lodged in the capsule had been destroyed by the epithelioid cells. In the latter they were found very rarely and only in immediate proximity to the main agar mass. They were not found in the peripheral regions of the capsule. Faintly acid-fast, beaded forms were occasionally seen free in the agar and at times, together with acid-fast globules, in the epithelioid and Langhans giant cells.

The same differences persisted *5 weeks* after inoculation between these two groups of animals. Caseation was very extensive in the normal animal. It was but slight or absent in the vaccinated. The bacilli were still multiplying within macrophages at the periphery of the capsule and in the central softened agar mass in the normal animal, whereas only rare isolated beaded forms persisted within the cells and in the thoroughly fibrin-permeated necrotic agar mass of the vaccinated rabbit.

The Draining Lymph Nodes.—

Except for some congestion of the capillaries in the superficial axillary nodes draining the agar focus in the normal animal the glands were entirely normal 1 day after inoculation. Both the marginal and intermediate sinuses were free of extraneous cells (Fig. 8). On the 3rd day large numbers of polymorphonuclears undergoing pyknosis and karyolysis were found in these sinuses and small numbers of tubercle bacilli were isolated from the node. 4 days later it yielded on culture 1,850 colonies, and large mononuclears with highly vesicular nuclei and reticulated cytoplasm, both isolated and in groups, some containing as many as 5 separate rod-shaped or coccoid forms of tubercle bacilli, were present in the cortex of the node. Agar globules were found in these cells, as well as in macrophages lying free in the peripheral sinuses. On the 2nd week after inoculation the multiplication of the bacilli had reached its height and young epithelioid cell tubercles, many containing long bacilli with bulbous ends, were found dispersed throughout the node, with frequent mitotic figures. There had been no significant change in the bacillary numbers in this node, 3 weeks later, when 31,900 colonies were cultured from it, and widespread epithelioid tubercles with rare giant cells and occasional caseous foci were found. Sparsely scattered bacilli were seen throughout.

In the B C G vaccinated rabbit in contrast, 1 day after inoculation the marginal sinus of the superficial axillary node was distended with fluid and contained large numbers of polymorphonuclears. These cells extended into the intermediate sinuses along the septa in the direction of the lymph flow and the medullary sinuses were filled with them. Macrophages with phagocytosed red blood cells and agar globules were also seen here (Fig. 9). In association with this evidence of the overflow of material from the agar focus into the lymph nodes, 30 colonies were

BCG reacted to the inoculation of virulent tubercle bacilli in agar by a greatly increased intensity of the vascular reaction. A more widespread edema resulted, and a more intense cellular infiltration of the tissues about the agar focus, in which mononuclears were prominently present on the *1st day* after inoculation. The agar was broken up into larger islands, the fibrin threads between them were coarser and more pronounced, and the cellular infiltration, including the mononuclears, have penetrated more deeply into the agar mass. Many polymorphonuclears already showed loss of granules and pyknosis of the nuclei. The carbon particles were aggregated in denser masses and the remaining agar coagulum was as a rule free from dispersed particles. The bacilli were often obscured by the carbon masses; otherwise, they were found in the same condition as in the normal animal.

On the *3rd day* the differences between the normal and vaccinated animals were of the same character. The endothelial cells of the blood vessels were swollen and the mononuclear cells had accumulated in the areolar tissue, as well as about the blood vessels, their cytoplasm was more developed, their nuclei were more vesicular and mitotic figures were more frequently found than in the normal animal. Active migration of the mononuclears toward the center of the agar mass was apparent at the periphery of the lesion. Tubercle bacilli were found, but with difficulty, throughout the lesion.

1 week after inoculation in the vaccinated animal the vascular congestion had subsided. Granulation tissue, agar-containing macrophages, and fibroblasts were more prominent than in the normal animal. Nodular collections of epithelioid cells had already appeared in the capsule, whereas the active multiplication of mononuclears was less intense than in the normal animal. Polymorphonuclears were much less prominent in the entire lesion. There was no definite zone of polymorphonuclear infiltration at the point of junction between the main agar mass and the capsule. The destruction of the polymorphonuclears had advanced much further than in the normal animal. Deep within the agar mass the fibrin bands between the agar islands were more prominent than the agar itself, so extensive was the deposition of fibrin between them. This was the height of the multiplication of the bacilli, such as it was, in the vaccinated animal. Yet the bacilli were difficult to find. They were rarely seen as isolated microorganisms in the granulation tissue, extremely rarely in the dense network of fibrin abutting against the main agar mass or free in the agar (Fig. 4). Here they could be found as isolated microorganisms or as occasional minute dense clumps without any radiations, in which the individual bacilli could not be distinguished. The bacilli were short, often coccoid, and at times had the appearance of spores, as suggested by their elliptical shape with a centrally located unstained portion in a thin shell of acid-fast material. Dark staining polar bodies were seen in some of the rods (Fig. 4). It was evident both from the cytological appearance and the more definite cultural results that while the bacilli multiplied to some extent in the vaccinated animal they were definitely inhibited in their growth, both when situated in the cells and when free in the agar, as compared with the normal animal.

agar saline suspension was sterile, whereas the agar plasma suspension contained 60,000 colonies.

A suspension of tubercle bacilli in agar, 10 mg. of which yielded on culture 2,200 organisms, was placed within a Chamberland L3 filter. The open end was effectively sealed, and the filter was buried between the muscles. At the end of 14 days the contents of the filter were sterile.

It is plain that in the absence of plasma or body fluids tubercle bacilli incorporated in agar die in 11 to 14 days both *in vivo* and *in vitro*.

In summary, the mechanism of immunity as revealed by this series of observations was as follows:

The inflammatory reaction in the vaccinated animal is more intense, which leads to a greater outpouring of cells and fluid into and about the agar focus containing the bacilli of reinfection. This increased accumulation of cells and fluid operates to sweep the bacilli into the draining lymph node more rapidly than in the normal animal, if the dose on reinfection is large (Figs. 8 and 9). On the other hand there are factors which tend mechanically to fix the bacilli to a greater degree at the portal of entry in the vaccinated than in the normal animals. There is the larger and denser barrier of fibrin about the agar focus as a whole, and the individual agar islands of the vaccinated animals (Figs. 2 and 4). The greater agglutination of particulate matter, as shown by the carbon particles in the agar focus of the vaccinated, would also tend to immobilize the tubercle bacilli. However, it would seem that these factors are less important than those which tend to inhibit the multiplication and enhance the destruction of the bacilli in the vaccinated animal. For it has been clearly demonstrated that an extracellular factor is present which inhibits the multiplication of the bacilli in the agar islands away from the cells in the vaccinated animal, whereas in the normal creature the multiplication of the bacilli in these regions is unhindered (Figs. 2 and 4). Furthermore there is a greater and more rapid mobilization of the mononuclears which engulf and destroy the tubercle bacilli in the vaccinated animal. This destruction is indicated by the more rapid formation of epithelioid and giant cell tubercles containing little if any bacilli, and is associated with the suppression of their growth as determined by culture. Thus both the extra- and intracellular factors operate to inhibit the growth

cultured. On the 3rd day the marginal sinus was practically free of polymorphonuclears, but numerous degenerating polymorphonuclears were found lying free or within swollen reticular cells in the cortex of the node. Small syncytia about agar globules were found in the medullary sinuses. Again 36 colonies were isolated. The bacilli, instead of multiplying unhindered as in the normal animal, had practically disappeared for the time, and 1 week after infection nodular collections of hypertrophied macrophages, some of which had assumed epithelioid forms, and some of which contained large agar globules, were found in the cortex of the node. However, this almost complete suppression of the invading bacilli in the lymph node of the vaccinated animal was not constant, and on the 14th day they were slowly increasing, associated with mature epithelioid cell formation and minute foci of caseation. In the 5th week this retarded growth of the bacilli continued associated with a liquefaction of the tuberculous foci.

The Lung.—

Bacilli reached the lung of the normal rabbit in the 1st week, while they were first cultured with regularity from the lung of the vaccinated rabbit in the 2nd week after inoculation, and then in significantly smaller numbers. In both instances the response in the lung was, from the very beginning, a combined interstitial and pneumonic reaction, as distinguished from the interstitial lesion that characterizes the initial response to a first infection by intravenous inoculation. In this connection the fact should be recalled that exudation into the alveoli of a normal animal is synchronous with the development of sensitivity to tuberculin and caseation as previously pointed out (7). In the present study the bacilli first reached the lung in significant numbers at a time when caseation had already started in the agar focus even in the normal animal. From then on, numerous conglomerate, extensively caseous, predominantly pneumonic lesions developed throughout the lung of the normal rabbit, with large numbers of microorganisms within the caseous foci and the epithelioid and giant cells within the alveoli. In the vaccinated animal, on the other hand, there developed rare, isolated lesions of the same nature, but with the distinction that the bacilli were present in very small numbers even in the intraalveolar epithelioid cells and the minute caseous foci. The latter observation was corroborated by the tremendous difference in the number of bacilli cultured from these lungs.

It was stated above that tubercle bacilli multiply in the acellular agar islands of the normal animal and that the fluid of the exudate penetrates these islands. To make certain that the growth in these foci was due to the fluid contained in the agar, experiments were performed of which the following will serve as examples.

An agar saline suspension containing 3,430 viable tubercle bacilli in 10 mg. of material was incubated at 37°C. simultaneously with a sample of agar mixed with normal rabbit plasma and containing 5,120 organisms. At the end of 11 days the

tubercle bacilli suspension containing 0.1 per cent trypan blue. An identical mixture without the bacilli was injected in the opposite foreleg.

In Table III is recorded the fate of the bacilli and the trypan blue in these two groups of rabbits. The colonies cultured from the lungs and spleen are omitted, for bacilli from the primary infection remained in these organs. In the lymph nodes, however, no bacilli remained from the primary infection, as was evident from the regularity of the rise and fall of the number of bacilli cultured, from the microscopic studies of these nodes and finally from the sterility of the lymph nodes not draining the agar focus. There was no significant difference in the number of bacilli cultured from the agar focus of the normal and tuberculous rabbits 1 day after inoculation of an agar suspension containing 10,900 tubercle bacilli in 10 mg. of material. As with B C G vaccinated rabbits there was practically complete suppression of multiplication of the bacilli of reinfection in the agar focus of the tuberculous rabbits, as distinguished from the massive growth of the microorganism in the normal animals. Again, as was noted in that series, the axillary lymph nodes draining the agar focus of the reinfected animal were already invaded by the bacilli on the 1st day after reinfection, while the lymph nodes draining the agar focus of the normal animal were still sterile at this time. There was however one important difference. While in the B C G vaccinated rabbits the bacilli still slowly and continuously increased in numbers up to the 5th week, although definitely retarded in their multiplication in the superficial draining lymph nodes; in rabbits having a virulent primary infection, on the other hand, the bacilli that invaded these nodes experienced only an ephemeral increase, which was soon suppressed. An identical result was obtained in another series of rabbits treated intravenously with large amounts of a heated suspension of Ravenel bacilli containing but a small number of living organisms and similarly reinfected later with an amount of bacilli similar to that of this series. In all instances the microorganisms that invaded the deeper nodes were destroyed practically completely.

It will be noted that the amount of trypan blue retained both in the agar focus and the draining lymph nodes was uniformly greater in the tuberculous than in the normal rabbit. This greater retention of dye at the site of reinfection was noted for both trypan blue and India ink in the BCG series described above, and was also observed, and to a greater extent, in actively tuberculous rabbits reinfected with melted agar containing India ink and tubercle bacilli. Furthermore the presence of bacilli in the mixture was not essential for this phenomenon, for it occurred, though perhaps to a less marked degree, when bacilli were not present in the injected agar.

An indication as to the mechanism of the more rapid dissemination of bacilli to the draining lymph nodes in the sensitized animal was apparent from the condition of the efferent lymphatic of the deep

and destroy the bacilli of reinfection. On the other hand in the normal animal the impotent polymorphonuclears persist and the mononuclears arrive later. Their capacity to destroy tubercle bacilli is markedly less than after vaccination, as indicated by the accumulation of bacilli within their cytoplasm, the more tardy formation of mature epithelioid tubercles and the vastly greater numbers of bacilli cultured. It is evident that in the normal animal both the extra- and intracellular factors operate to enhance the growth and disseminate the bacilli.

The proliferation of tuberculous tissue, its caseation and the massive multiplication of the bacilli, both intracellularly and free in the agar, are most prominent in the normal animal, whereas the reaction to the agar in the focus with the formation of foreign body giant cells and fibrous tissue is the most conspicuous features of the agar capsule in the vaccinated animal (Fig. 7).

It is the enhanced capacity of the mononuclear phagocytes of the B C G vaccinated rabbit to destroy tubercle bacilli that is the determining factor in the dissemination of the disease to the draining lymph nodes and internal organs. For when the bacilli reach these structures, even if they penetrate them more rapidly than in the normal animal, the phagocytes soon destroy them, as indicated by the more rapid epithelioid cell formation associated with suppression of their growth, as demonstrated by culture.

The Host-Parasite Relationships in Normal and Tuberculous Rabbits

It is generally held that a greater immunity to reinfection to tuberculosis is afforded by a virulent primary infection than by one of low virulence. With the hope of throwing further light on the mechanism of immunity the experiments described above were repeated on a series of animals primarily infected with virulent tubercle bacilli and reinfected as previously with melted agar containing virulent bacilli.

The Fate of the Bacilli.—

In order to produce a slowly progressive tuberculosis with minimal involvement of the lymphatic system a group of rabbits were vaccinated by an intravenous injection of 1.0 mg. of living B C G. 60 days later they received 0.0001 mg. of the Ravenel strain of bovine type tubercle bacilli by the same route. 97 days after the last injection these tuberculous rabbits, together with a group of normal animals, received subcutaneously in the right foreleg 5 cc. of an agar-bovine

axillary node. This vessel was greatly distended and was colored with trypan blue in the tuberculous animal 1 day after reinfection. A similar observation is recorded below in another group of tuberculous animals. This evidence of increased lymph flow from the site of inoculation was not encountered in normal animals.

In Table IV are recorded the results of a similar experiment with the difference that the reinfecting dose was about ten times less than that in the preceding series.

All the tuberculous rabbits in this series except rabbit 9-6 received 1.0 mg. B C G intravenously, followed 99 days later by 0.001 mg. of the Ravenel strain by

TABLE IV

The Fate of Virulent Bovine Tubercle Bacilli in a Localized Agar Focus and Their Dissemination in the Body in Normal and Tuberculous Rabbits (Series 4)

Agar suspension	Interval after inoculation	Rabbit No.		Agar focus		Superficial axillary node		Deep axillary node		Popliteal node	
		Normal	Tuberculous	Normal	Tuberculous	Normal	Tuberculous	Normal	Tuberculous	Normal	Tuberculous
1,070	1 day	21-2	9-6	4,200	700	0	10,216*	0	0†	—	0
	1 wk.	21-1	F-1	3,000	100	47	0	80	‡	—	0
	2 wks.	21-5	F-2	265,000	2,400	1,520	0	760	1	—	0
	4 wks.	21-4	G-1	20,000	230	12,500	0	1,400	1	—	0

* Signs of acute inflammation; no tuberculous changes.

† The lymph trunk between the deep axillary node and the axillary vein is distended and faintly blue.

‡ 106 colonies were cultured from this node; there was a large regressive tubercle undergoing organization; obviously due to the primary infection.

the same route. 34 days after the last injection, these rabbits, together with a group of normal animals, received a mixture of agar, bovine tubercle bacilli and trypan blue subcutaneously over the right chest wall. Rabbit 9-6 received its subcutaneous reinfection 72 days after an intravenous inoculation of 0.001 mg. of the Ravenel bacilli.

It will be noted that the control popliteal lymph nodes were sterile in each of the reinfected tuberculous animals. Again the axillary nodes draining the agar focus of the normal animal were sterile 1 day after inoculation, but large numbers of tubercle bacilli were isolated from the superficial axillary nodes draining the agar focus of the

TABLE III

The Fate of Virulent Bovine Tubercle Bacilli and Trypan Blue in a Localized Agar Focus and Their Dissemination to the Draining Lymph Nodes in Normal and Tuberculous Rabbits (Series 3)

Agar suspension	Interval after inoculation	Rabbit No.		Agar focus				Superficial axillary node				Deep axillary node			
				Normal		Tuberculous		Normal		Tuberculous		Normal		Tuberculous	
		Normal	Tuber-culous	Colonies	Trypan blue	Colonies	Trypan blue	Colonies	Trypan blue	Colonies	Trypan blue	Colonies	Trypan blue	Colonies	Trypan blue
10,900	1 day	6	14-0	10,000	+	12,000	+	0	±	0	+	0	±	16	+±*
	3 days	7	14-1	76,400	+	1,800	++	39	±	164†	+	180	±	46	+
	1 wk.	8	14-2	69,600	+	23,900	+++	5,742	±	3,478	++	2,654	±	5	±
	2 wks.	9	14-3	260,000	±	14,200	+	9,300	+	242	+++	8,400	±	5	+++
	4 wks.	10	14-4	120,000	±	1,260	—	16,600	0	222	+	—	0	8	±

The intensity of coloration of the agar focus and draining lymph nodes is graded as follows: 0, no blue; ±, faintly blue; +, pale blue; ++, moderately blue; and +++, deep blue.

* Lymph trunk between deep axillary node and axillary vein distended to 3 mm. in diameter and faintly blue.

† Inguinal nodes 0.

reached the draining lymph nodes before those did in the normal animals. It is noteworthy that in the series in which the inflammation of reinfection was not intensified the dissemination of bacilli to the draining lymph nodes was not accelerated, nor did these nodes show any evidence of overflow of leukocytes into the nodes from the agar focus, which was so conspicuous in the other series.

As against this the factors that act mechanically to fix bacilli *in situ* were more pronounced in the tuberculous than in the B C G vaccinated animals. The carbon was agglutinated in denser masses (Figs. 12 and 13). The phagocytosis of trypan blue by the macrophages that surrounded the agar focus was more intense, and the individual globules of dye were much coarser than those in the B C G vaccinated rabbits. Likewise the agglutination of tubercle bacilli was more pronounced in this group and was particularly evident in the series of rabbits treated with a mixture of killed and a few living virulent tubercle bacilli which, incidentally, showed specific agglutinins in a 1:64 dilution of their serum before reinfection. The fibrin barrier about the agar focus, while much more pronounced than in normal animals, was not consistently more marked in the tuberculous than in the B C G series.

The same humoral inhibitory influence was apparent on the growth of the bacilli in the acellular agar as had been noted in the B C G series (Figs. 3 and 5). The mobilization of mononuclears was more rapid and their physiological development was more accelerated than in the B C G series. Also the necrosis and rapid lessening in number of the polymorphonuclears was conspicuous in the reinfected rabbits. The increased capacity of the phagocytes to destroy tubercle bacilli was clearly more in evidence in the tuberculous animals, and epithelioid cell formation was much further advanced at the end of the 1st week. There resulted from all these circumstances a more pronounced foreign body and fibrous tissue reaction in the capsule surrounding the agar focus of the tuberculous rabbits. Similar observations were made in the lymph nodes. Large masses of agar enclosed within syncytia could be found in the practically sterile nodes of the reinfected tuberculous animals (Fig. 11), whereas in the normal animals these syncytia contained large numbers of tubercle bacilli both microscopically and on culture (Fig. 10).

reinfected animal. These nodes were definitely stained with trypan blue and the efferent lymphatic from the deeper node was slightly distended and contained faintly bluish lymph. There were no microscopic tuberculous changes in this node but there was a great accumulation of polymorphonuclears and red cells in the sinuses. All this makes it certain that the bacilli were not residual bacilli from the primary infection. After this period the draining lymph nodes in the reinfected animals were practically sterile, while bacilli were present in increasingly large numbers in the normal animal. Identical observations were made in other tuberculous rabbits likewise reinfected with small doses. If the dose was small enough, so that the agar inoculum contained less than 500 bacilli per 10 mg. of substance, they never reached the draining lymph nodes of the reinfected rabbit. In the agar focus of these rabbits only small numbers of tubercle bacilli persisted after 4 weeks.

These results again emphasize the greater immunity afforded by a virulent primary infection as compared with that conferred by a primary infection of low virulence. For even with a small reinfecting dose the bacilli were still increasing slowly in the superficial axillary nodes of BCG vaccinated rabbits at the end of 4 weeks, whereas in tuberculous rabbits the bacilli of reinfection were completely destroyed in the draining lymph nodes at the end of the 1st week.

The Reactions of the Host.—

What is the host expression of this greater immunity of rabbits affected by a virulent tuberculosis as compared with that of animals vaccinated with BCG? The difference is quantitative rather than qualitative. There was no conspicuous difference in the intensity of the initial inflammation to reinfection in these two groups. In fact in one series of reinfected rabbits, treated intravenously with large amounts of heated bacilli containing a few living virulent microorganisms, the initial inflammation on reinfection was actually less than that of the normal animal. However in the other two series reported here in detail the intensity of inflammation was much greater in the tuberculous animals than in the normal controls. This was associated with evidence of an increased lymph flow from the infected agar focus, which swept the bacilli out more rapidly so that they

tion has significance under other conditions than those afforded by the introduction of agar would seem indicated by the studies of Woodruff (8) on omental spreads of guinea pigs. He noted freely growing bacilli immediately adjacent to or some little distance from the cells of normal animals, a picture absent in reinfected animals.

The agar focus demonstrated in striking fashion extracellular factors which would tend to immobilize and limit the spread of the surviving bacilli in the immunized animal. Carbon particles and trypan blue were retained more effectively at the site of inoculation in the reinfected than in the normal animal. The retention of carbon at the site of introduction was obviously due to the clumping of these into dense large masses whose mobility in the tissues was obviously retarded (Fig. 13). In the normal animal this clumping was much less in evidence (Fig. 12).

Pagel (9) noted a greater retention of carbon at the site of reinfection than at the site of primary injection of mixtures of tubercle bacilli and carbon into guinea pigs. The phagocytosed trypan blue particles of our experiments were much coarser in the immunized than in the normal animal. Somewhat parallel observations were made with tubercle bacilli growing in the acellular agar. In the normal animal they accumulated as freely dispersed rods or as large loose colonies (Figs. 2 and 3). In the immunized animal they either persisted as isolated inactive individuals (Fig. 4) with structures often suggesting spore forms or, if colonies did form, they were minute and very dense so that the individual rods could not be distinguished (Fig. 5). This *in vivo* agglutination, as well as the denser and broader fibrin barrier thrown about the individual agar islands and about the agar mass as a whole, tended to immobilize the bacilli. *In vivo* agglutination of bacteria in the immunized animal has been noted by Rich with pneumococci (10) by Cannon and Pacheco with staphylococci (11) and by Woodruff with tubercle bacilli (8).

The various extracellular factors are effective in confining the bacilli at the site of reinfection when small doses are introduced. This was found to be true in tuberculous rabbits reinfected with small doses. However with larger doses and in association with a greatly intensified inflammation of reinfection all these factors tending to immobilize the bacilli are overcome by the greatly increased flow of lymph, which

DISCUSSION

By introducing agar impregnated with tubercle bacilli in the tissues of an animal conditions are produced which afford an excellent opportunity for studying extracellular effects on the growth of the parasite. For the body fluids penetrate the agar masses, whereas the cells invade the solid agar islands slowly. By this means it was shown that in the normal animal the bacilli grew unhindered in the acellular agar at a great distance from the cells (Figs. 2 and 3). In the vaccinated or tuberculous animal on the other hand a marked inhibition of their growth was evident (Figs. 4 and 5). If the body fluids are prevented from entering the agar mass, as was accomplished by burying Chamberland L3 filters filled with agar and tubercle bacilli between the muscles, the bacilli die completely in 14 days. Tubercle bacilli impregnated in saline agar mixtures such as were used for inoculation, and left at incubator temperature die in 11 days. Since even in the vaccinated animals tubercle bacilli were cultured from the agar it is evident that their persistence was due to the fluids that penetrated the agar. It would appear, therefore, that in a normal animal the fluids penetrating the agar focus support the growth of tubercle bacilli, whereas the fluid from a vaccinated or tuberculous animal is so altered that it permits slight or no multiplication of the bacilli.

The fibrin barrier thrown about the agar focus in the immunized animal is more pronounced than that about the focus of the normal animal (Figs. 2 and 4). It is conceivable therefore that the differences just noted may be attributable in part to a greater hindrance to the penetration of nutritive substances into the agar islands of the immunized animal. However, in the normal animal at the height of the multiplication of the bacilli, there is a vast necrotic zone impregnated with fibrin separating the main agar mass from the capsule; yet the interposition of this barrier does not hinder the massive multiplication of the bacilli in the acellular agar, while in the immunized rabbit the bacilli fail to multiply in immediate juxtaposition to the living macrophages surrounding and infiltrating the main agar mass. It would seem therefore that the inhibitory influence of the fluid penetrating the agar of the vaccinated animal must be attributed to an inherent difference in its constitution. This demonstrates a rôle of humoral bacteriostatic substance in immunity to tuberculosis. That the observa-

tion of reinfection will bring about a more rapid dissemination of the bacilli, if man reacts as a rabbit does; but even in such case as soon as the invading bacilli reach a new territory the forces of immunity, both humoral and cellular, tend to fix and destroy them.

It has been demonstrated in the course of the present work that vaccination of rabbits with BCG affords a pronounced protection against a virulent infection. This is not surprising in view of the fact that the BCG produces a disease in no way different fundamentally, both from the standpoint of the host and that of the parasite, from that of a virulent tuberculosis, with the all important exception that the lesions regress very rapidly and soon disappear completely, while the bacilli of the vaccination disappear with the exception of an occasional lingering microorganism in the lymph nodes (7). Many experimental and clinical studies point in the same direction.

In accord with our previous studies (4) the bacilli of reinfection are more effectively destroyed by an animal with residual lesions from a primary infection than by one without lesions. It is important to emphasize these observations, for recently Selter and his coworkers (15) in an attempt to repeat our experiments, came to the conclusion that a progressive primary tuberculosis exercises no greater inhibitory effect on the bacilli of reinfection, than a slight nonprogressive primary infection. An analysis of their experiments reveals that the guinea pigs which they used for the reinfection experiment had received 1 month previously a dose and strain of tubercle bacilli which killed them within 2 to 3 months with generalized tuberculosis. Only 2 to 5 days after reinfection these pigs showed macroscopic tubercles in the lung, liver and spleen. These lesions Selter himself attributed to the primary infection. Under these conditions they found no significant difference in the number of bacilli cultured from the reinfected and the control animals. It is obvious that some, at least, of the bacilli cultured from these reinfected animals were most likely from the primary infection and not the reinfection, and, while these authors state that it is desirable to determine the number of bacilli remaining in the organs from the primary infection, this consideration did not deter them from drawing a conclusion that seems questionable.

The greater immunity afforded by a virulent primary infection expressed itself as a quantitative increase of those factors which operate

sweeps the polymorphonuclear leukocytes and agar particles, some of which contain bacilli, more rapidly into the draining nodes than in a normal animal (Figs. 8 and 9).

Field, Drinker and White (12) found that a sterile inflammation produced by the application of heat caused a markedly increased flow of lymph. Hudack and McMaster (13) noted that in the human skin recently inflamed by heat, by ultraviolet light or by bacterial vaccine or toxin the lymphatic capillaries become far more permeable to vital dyes than in the normal skin. Menkin (14) found that the fixation of trypan blue at the site of inflammation depends upon the character of the irritant. The powerful necrotizing agent of *S. aureus* produces rapid fixation. Mild irritants on the other hand produce only delayed fixation. It is obvious that the inflammation caused by the tubercle bacillus of reinfection in the rabbit belongs in the latter category.

Despite the lack of immediate fixation in the vaccinated animal immunity is none the less effective. For the other and perhaps more important agent in immunity to tuberculosis, the increased capacity of the mononuclear phagocytes to destroy tubercle bacilli, asserts itself at the site of reinfection as well as in the draining lymph nodes. The polymorphonuclear leukocytes which are unable to destroy tubercle bacilli soon die and are replaced by the rapidly mobilized mononuclear phagocytes in the immune rabbit, in whose interior the bacilli are rapidly destroyed with accelerated formation of epithelioid tubercles (Fig. 7). In the normal animal the impotent polymorphonuclears persist and the mononuclears are tardy in their appearance. Moreover their capacity for destroying tubercle bacilli is slight, so that the microorganisms at first accumulate in large numbers within their cytoplasm (Figs. 6 and 10).

These observations permit a definite view on the question of the rôle of allergy in tuberculosis. With small doses on reinfection, such as those from exogenous sources in man under natural conditions, the immunity acquired from the primary lesion, which accompanies the allergy under these conditions, brings about the fixation of the bacilli at the portal of entry and the inhibition of their multiplication as described above. With larger doses however, such as can be derived only from endogenous spread, the heightened inflamma-

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EXPLANATION OF PLATES

All sections were prepared from tissues stained either with hematoxylin-eosin or by the Ziehl-Neelsen method, and counterstained with hematoxylin. The magnifications given are approximate.

PLATE 52

FIG. 1. The agar focus of normal rabbit 3 of B C G series 2, 1 day after inoculation; 9,000 colonies were isolated. The agar is broken up into small particles with coagulated fibrillar strands between them. Polymorphonuclears infiltrate these spaces. × 100.

FIG. 2. The agar focus of normal rabbit 5 of B C G series 2, 2 weeks after inoculation; 492,000 colonies were isolated. Large, loose actively growing colonies are present in the acellular agar. Vast numbers of long discrete rods are swarming in the necrotic exudate separating the agar islands. × 200.

FIG. 3. The agar focus of normal rabbit 9 of tuberculous series 3, 2 weeks after inoculation; 260,000 colonies were isolated. Loose aggregates of individual tubercle bacilli are present in the agar at a great distance from cells of any kind. × 200.

FIG. 4. The agar focus of vaccinated rabbit 12-2 of B C G series 2, 2 weeks after inoculation of the same suspension of bacilli as in rabbit 5 of Fig. 2; 3,000 colonies were isolated. The agar islands are separated by a very prominent dense fibrinous exudate. No tubercle bacilli are found in the agar. In the center of the figure, as indicated by the arrow, a tubercle bacillus with a polar body can be seen in the fibrinous exudate. × 200.

in the mechanism of resistance of animals immunized with a less virulent infection.

CONCLUSIONS

1. There is an extracellular factor which inhibits the growth of tubercle bacilli in immunized rabbits.

2. Extracellular factors localize carbon particles, trypan blue and tubercle bacilli at the site of introduction to a greater extent in the immunized than in the normal animal.

3. This greater fixation is brought about by an increase in the density and extent of the fibrin barrier formed about the focus of the immunized animal. The more pronounced *in vivo* agglutination of tubercle bacilli and carbon particles in the vaccinated or tuberculous rabbit also tends to immobilize them in the tissues.

4. The growth inhibitory and localizing agents are effective in the fixation of small doses on reinfection at the portal of entry.

5. With large doses on reinfection, the increased lymph flow resulting from the intensified inflammation in the immunized animal brings about a more rapid dissemination of the bacilli to the draining lymph nodes than in the normal animal.

6. The most significant factor in immunity is the increased capacity of the rapidly mobilized mononuclear phagocytes to destroy tubercle bacilli. The impotent polymorphonuclear leukocytes quickly disappear from the site of reinfection.

7. The invading bacilli that reach the draining lymph nodes of the immunized animal are retarded in multiplication or destroyed by these phagocytes.

8. Vaccination of rabbits with BCG brings into play the factors tending to immobilize the bacilli of reinfection, inhibit their growth and destroy them with a resulting significant immunity.

9. A virulent primary infection affords a greater immunity than one of low virulence and the host reactions are expressed by a quantitative increase in those immunity factors which operate in a vaccinated animal.

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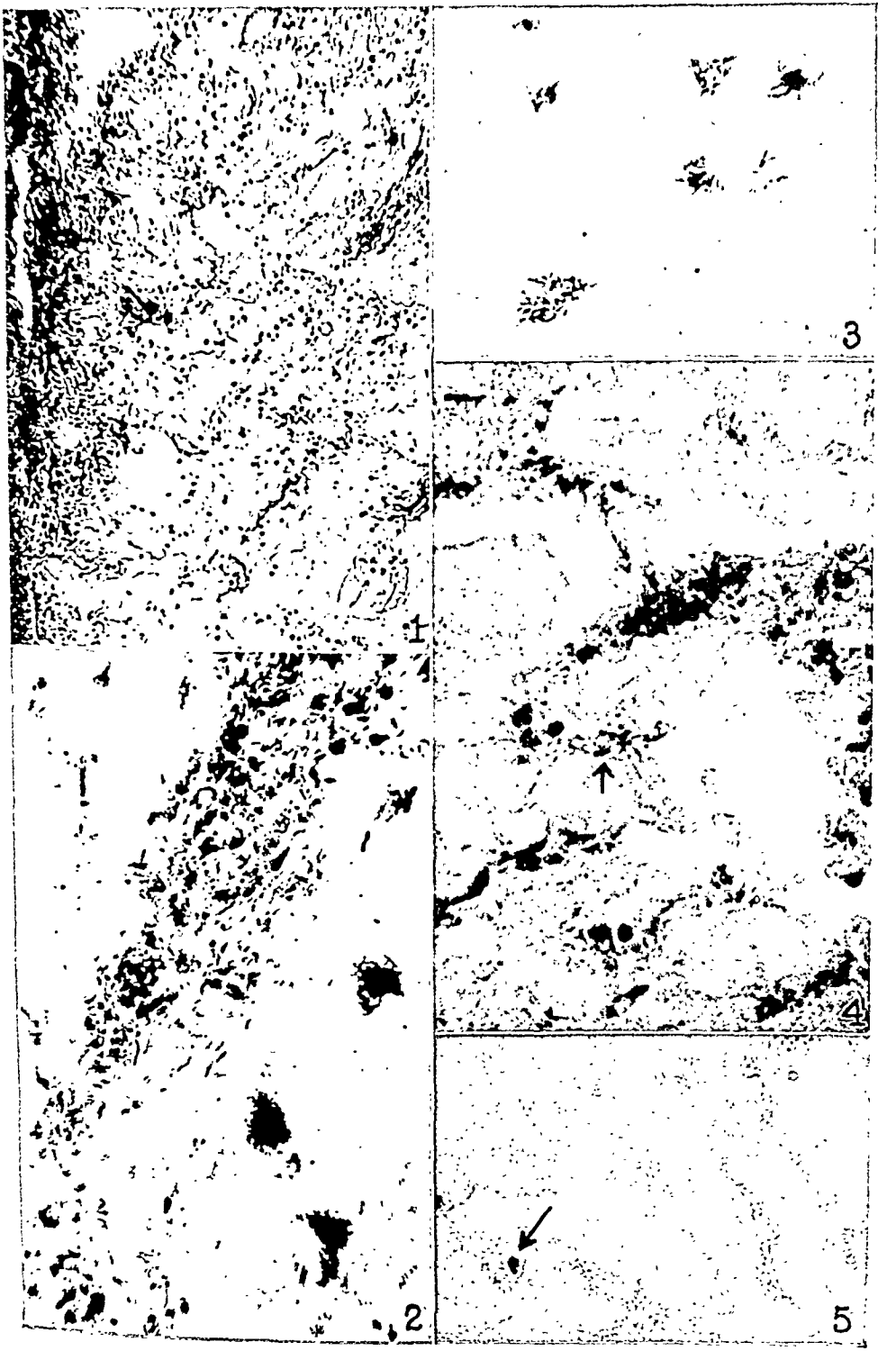
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FIG. 5. The agar focus of tuberculous rabbit 14-3 of tuberculous series 3, 2 weeks after reinfection with the same suspension of bacilli as in rabbit 9 of Fig. 3; 14,200 colonies were isolated. A minute dense clump of short bacilli in the acellular agar is indicated by the arrow. × 200.

PLATE 53

FIG. 6. The capsule surrounding the agar focus of normal rabbit 5 shown in Fig. 2; 492,000 colonies were isolated. A nodule of young epithelioid cells containing numerous tubercle bacilli surrounded by actively multiplying mononuclears. × 200.

FIG. 7. The capsule surrounding the agar focus shown in Fig. 4 of the BCG vaccinated rabbit 12-2; 3,000 colonies were isolated. Foreign body giant cells and mature epithelioid cells are prominent. × 200.

FIG. 8. The superficial axillary lymph node draining the agar focus of normal rabbit 3 of B C G series 2, 1 day after inoculation; no tubercle bacilli were obtained on culture. The lymph node is normal and in a resting state. The intermediary sinuses are free of infiltrating cells. × 200.

FIG. 9. The superficial axillary lymph node draining the agar focus of vaccinated rabbit 13-9 of B C G series 2, 1 day after inoculation; 30 colonies were isolated. The intermediary sinuses are engorged with fluid and polymorphonuclear leukocytes; macrophages and red blood cells are to be seen. × 200.

PLATE 54

FIG. 10. The deep axillary node draining the agar focus of normal rabbit 9 of tuberculous series 3, 2 weeks after inoculation; 8,400 colonies were isolated. Large giant cells enclosing agar globules, and young epithelioid cells undergoing the first stage of caseation with numerous tubercle bacilli. × 200.

FIG. 11. The deep axillary node draining the agar focus of tuberculous rabbit 14-2 of tuberculous series 3, 1 week after reinfection with the same suspension of bacilli as in rabbit 9 of Fig. 10; 5 colonies were isolated. A syncytium surrounding a large agar mass. No tubercle bacilli could be found. × 200.

FIG. 12. The agar focus of normal rabbit Ar, 13 days after inoculation of an agar, India ink and tubercle bacilli suspension; 155,400 colonies were isolated. The bacilli and carbon particles are scattered in the acellular agar. The agglutinated masses of carbon are slight. × 200.

FIG. 13. The agar focus of tuberculous rabbit 14-4, 13 days after reinfection with the same suspension of agar, tubercle bacilli and India ink as in rabbit Ar of Fig. 12; 2,700 colonies were isolated. The carbon is clumped in large dense masses. In the center of the field as indicated by the arrow are 2 dense clumps of tubercle bacilli surrounded by necrotic cells. One of the latter has the silhouette of a poodle dog. × 200.

(Lurie: Mechanism of immunity in tuberculosis)

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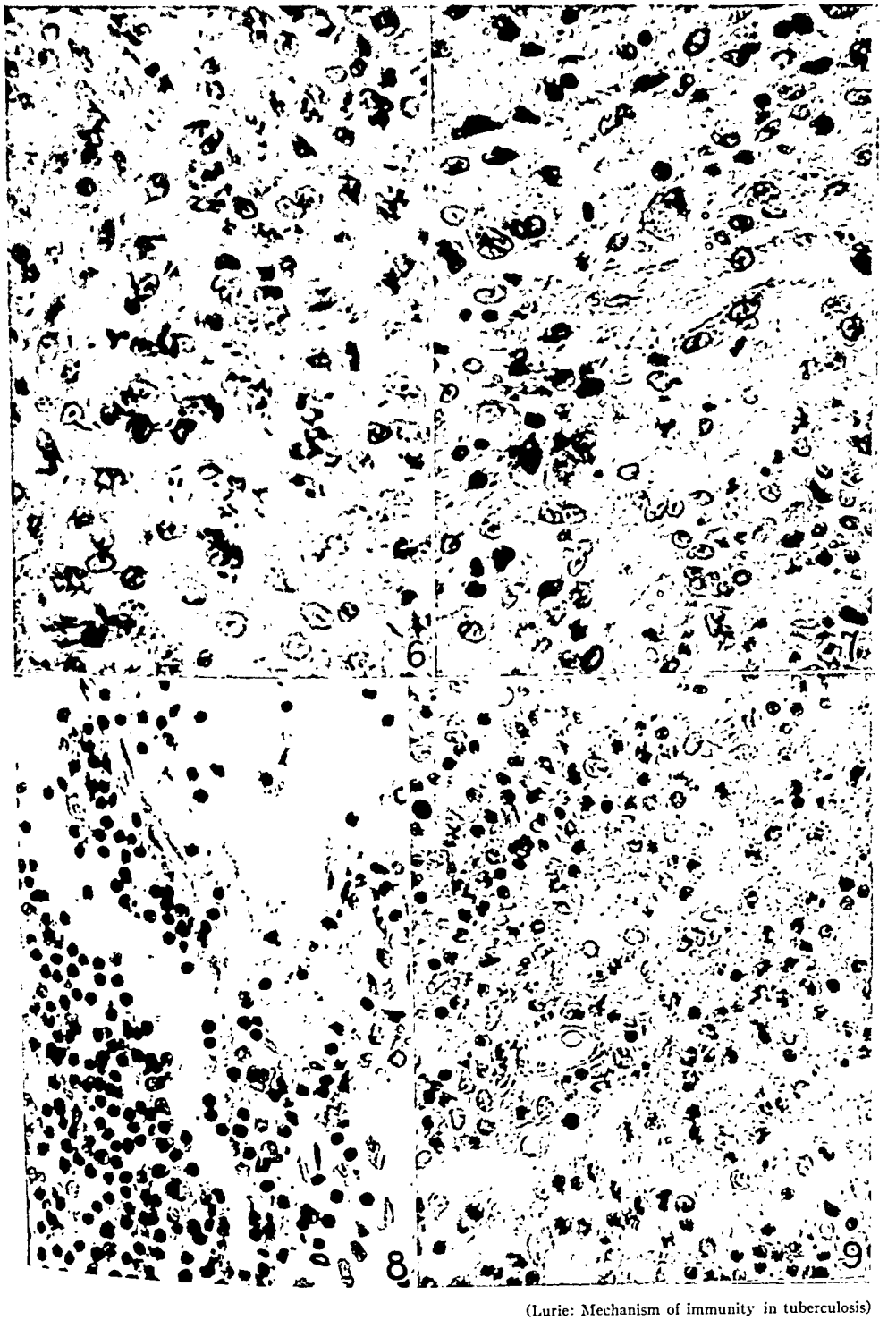

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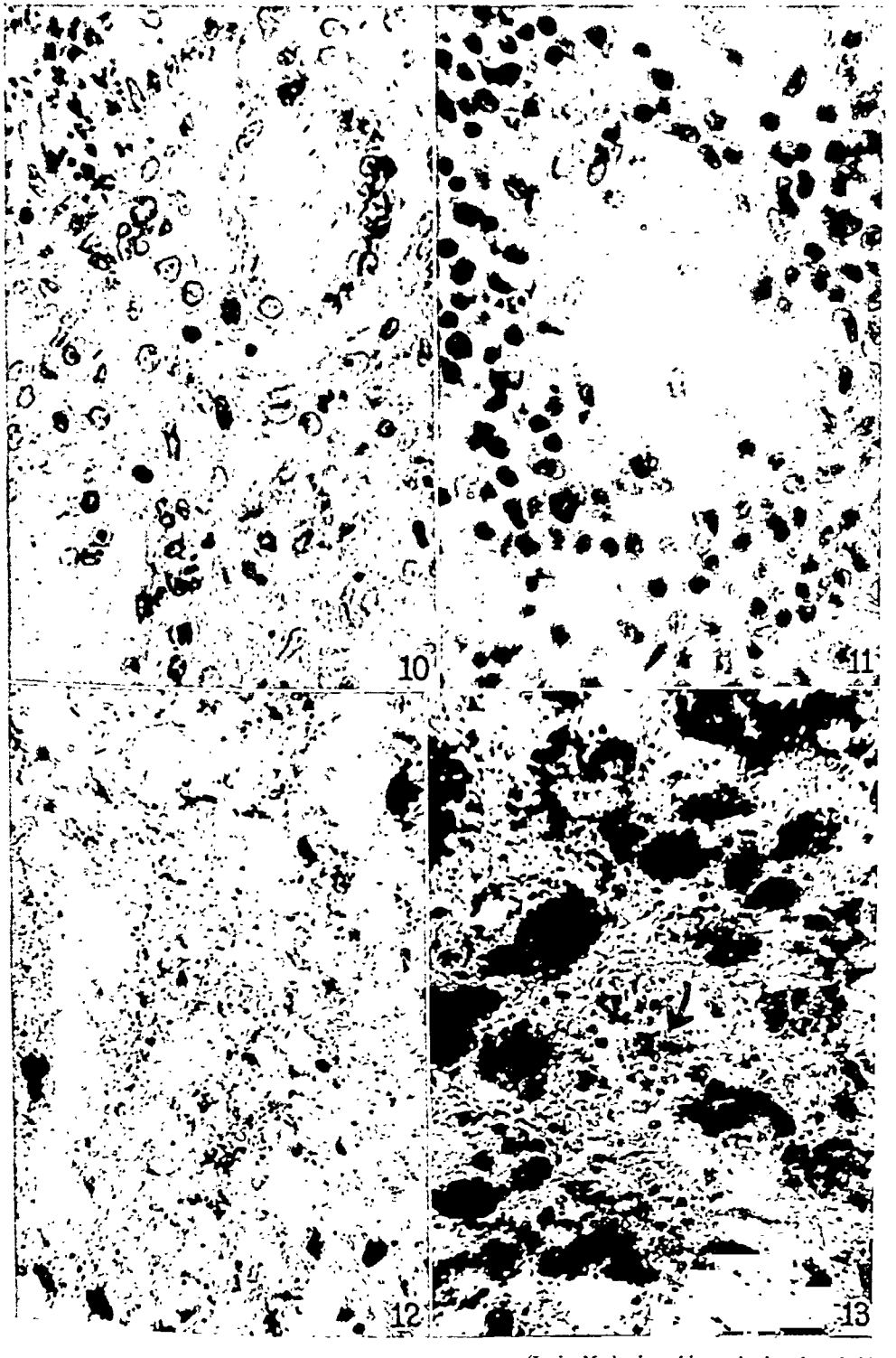

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